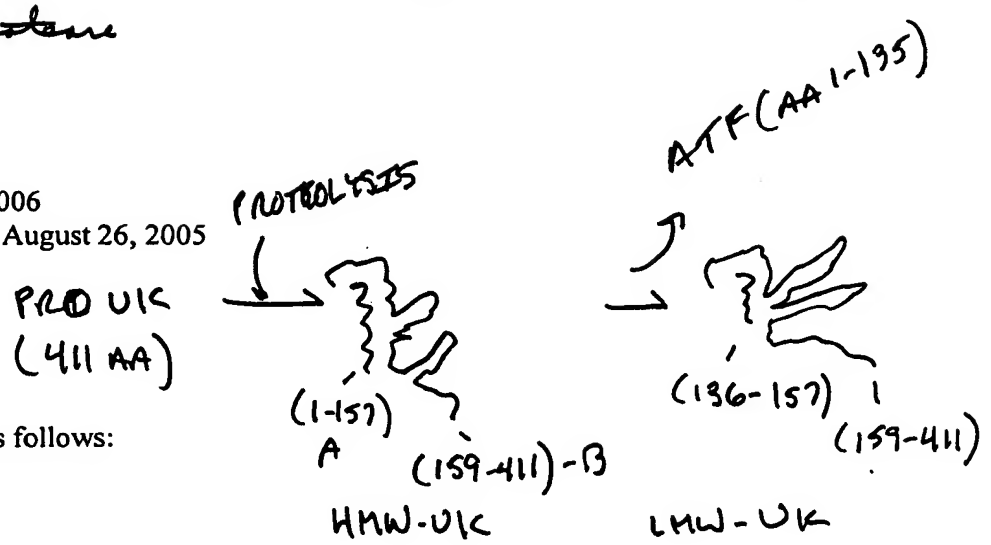
ATF HMW-UK (AA 1-135)

-UK = serine protease

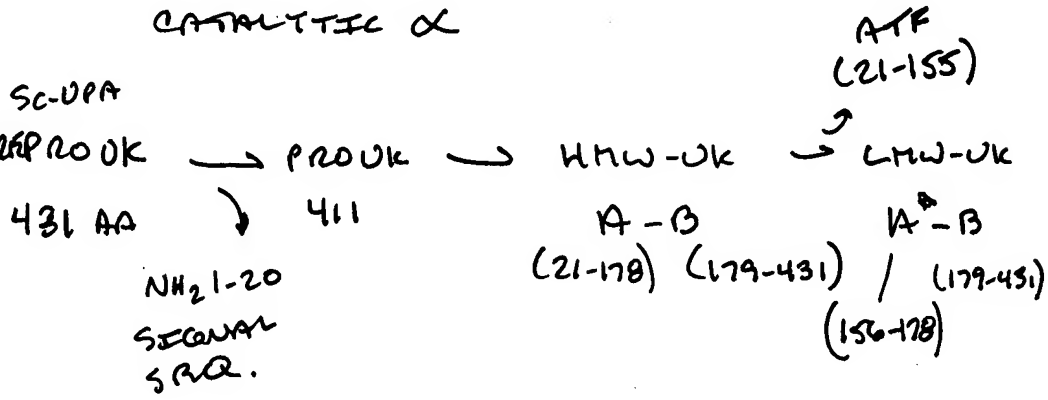


Listing of Claims:

Please **amend** the claims as follows:

Claim 1 (Cancelled)
Claim 2 (Cancelled)
Claim 3 (Cancelled)
Claim 4 (Cancelled)
Claim 5 (Cancelled)
Claim 6 (Cancelled)
Claim 7 (Cancelled)
Claim 8 (Cancelled)
Claim 9 (Cancelled)
Claim 10 (Cancelled)
Claim 11 (Cancelled)
Claim 12 (Cancelled)
Claim 13 (Cancelled)
Claim 14 (Cancelled)
Claim 15 (Cancelled)
Claim 16 (Cancelled)
Claim 17 (Cancelled)
Claim 18 (Cancelled)
Claim 19 (Cancelled)
Claim 20 (Cancelled)
Claim 21 (Cancelled)

AA 1-135 ARE NOT REQUIRED FOR CATALYTIC ∝



ATF COMPRESSES: KRINGLE DOM.; KRINGLE DOM.; + CD87 PROTEASE DOMAIN

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6,248,715
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NO DATA

Claim 22 **(Cancelled)**

Claim 23 **(Cancelled)**

Claim 24 **(Cancelled)**

Claim 25 **(Cancelled)**

Claim 26 **(Cancelled)**

ART

Claim 27 **(Previously presented)** An anti-HIV-1 pharmaceutical composition for injection comprising:

an amino-terminal fragment of the high molecular weight urokinase-type plasminogen activator (HMW-uPA) as an active component, the fragment being contained in a sterile aqueous or non-aqueous medium, wherein the fragment comprises amino acids 21-155 of the prepro-urokinase (sc-uPA) and does not extend beyond amino acid 178 of the sc-uPA.

→ [1-135]

↳ NT?
↳ ART 1-135 [155]

Claim 28 **(Cancelled)**

Claim 29 **(Cancelled)**

Claim 30 **(Cancelled)**

Claim 31 **(Cancelled)**

Claim 32 **(Cancelled)**

Claim 33 **(Cancelled)**

Claim 34 **(Cancelled)**

Claim 35 **(New)** A method for treating an HIV-1-infected human for suppression of reproduction of HIV-1 in the human comprising:

injecting the human with an HIV-1 reproduction-suppressive amount of an anti-HIV-1 pharmaceutical composition for injection comprising an amino-terminal fragment of the high molecular weight urokinase-type plasminogen activator (HMW-uPA) as an active component,

the fragment being contained in a sterile aqueous or non-aqueous medium, wherein the fragment comprises amino acids 21-155 of the prepro-urokinase (sc-uPA) and does not extend beyond [1-155] amino acid 178 of the sc-uPA.

Claim 36 **(New)** The method according to claim 35, wherein the fragment consists of amino-acids 21-155 of the sc-uPA.

Claim 37 **(New)** A method for treating an HIV-1-infected human for suppression of reproduction of HIV-1 in the human comprising injecting the human with an HIV-1 reproduction-suppressive amount of an anti-HIV-1 pharmaceutical composition for injection comprising an amino-terminal fragment of the high molecular weight urokinase-type plasminogen activator (HMW-uPA) as an active component, the fragment being contained in a sterile aqueous or non-aqueous medium, wherein the fragment contains an EGF-like domain, a Kringle domain and a urokinase receptor binding domain of the high molecular weight urokinase-type plasminogen activator (HMW-uPA) and no portion of the B-chain of the HMW-uPA.

Claim 38 **(New)** The method according to claim 35, wherein the aqueous medium is selected from the group consisting of water and an aqueous solution of one or more

pharmaceutically acceptable inert solutes and the non-aqueous medium is selected from the group consisting of polyalcohols, vegetable oils and organic esters.

Claim 39 **(New)** The method according to claim 36, wherein the aqueous medium is selected from the group consisting of water and an aqueous solution of one or more pharmaceutically acceptable inert solutes and the non-aqueous medium is selected from the group consisting of polyalcohols, vegetable oils and organic esters.

Claim 40 **(New)** The method according to claims 37, wherein the aqueous medium is selected from the group consisting of water and an aqueous solution of one or more pharmaceutically acceptable inert solutes and the non-aqueous medium is selected from the group consisting of polyalcohols, vegetable oils and organic esters.

Claim 41 **(New)** A method for treating an HIV-1-infected human for suppression of reproduction of HIV-1 in the human comprising:

transnasally or transpulmonarily applying to the human an anti-HIV-1 pharmaceutical composition for transnasal or transpulmonary application in the form of a dry powder consisting of an amino-terminal fragment of the high molecular weight urokinase -type plasminogen activator (HMW-uPA) as an active component and a carrier, wherein the fragment comprises

amino acids 21-155 of the prepro-urokinase (sc-uPA) and does not extend beyond the amino acid 178 of the sc-uPA.

Claim 42 **(New)** The method according to claim 41, wherein the fragment consists of amino-acids 21-155 of the sc-uPA.

Claim 43 **(New)** A method for treating an HIV-1-infected human for suppression of reproduction of HIV-1 in the human comprising:

transnasally or transpulmonarily applying to the human an anti-HIV-1 pharmaceutical composition for transnasal or transpulmonary application in the form of a dry powder consisting of an amino-terminal fragment of the high molecular weight urokinase-type plasminogen activator (HMW-uPA) as an active component and a carrier, wherein the fragment contains an EGF-like domain, a Kringle domain and a urokinase receptor binding domain of the high molecular weight urokinase-type plasminogen activator (HMW-uPA) and no portion of the B-chain of the HMW-uPA.

Claim 44 **(New)** The method according to claim 41, wherein the carrier is at least one compound selected from the group consisting of monosaccharides, disaccharides, polysaccharides, sugar alcohols, hydroxypropylccellulose, hydroxypropylmethylcellulose,

methylcellulose, hydroxyethylcellulose, polyvinylpyrrolidone, polyvinyl alcohol, nonionic surfactants, gelatin, casein, polyethylene glycol and hydrogenated lecithin.

Claim 45 **(New)** The method according to claim 42, wherein the carrier is at least one compound selected from the group consisting of monosaccharides, disaccharides, polysaccharides, sugar alcohols, hydroxypropylccellulose, hydroxypropylmethylcellulose, methylcellulose, hydroxyethylcellulose, polyvinylpyrrolidone, polyvinyl alcohol, nonionic surfactants, gelatin, casein, polyethylene glycol and hydrogenated lecithin.

Claim 46 **(New)** The method according to claim 43, wherein the carrier is at least one compound selected from the group consisting of monosaccharides, disaccharides, polysaccharides, sugar alcohols, hydroxypropylccellulose, hydroxypropylmethylcellulose, methylcellulose, hydroxyethylcellulose, polyvinylpyrrolidone, polyvinyl alcohol, nonionic surfactants, gelatin, casein, polyethylene glycol and hydrogenated lecithin.

References

1 Parmley, S. F. and Smith, G. P. (1988) *Gene* 73, 305–318
2 Ladner, R. C. and Guterman, S. K. (1990) *Patent Appl.* WO90/02809
3 Ladner, R. C., Guterman, S. K., Roberts, R. L., Markland, W., Ley, A. C. and Kent, R. B. (1993) *US Patent No.* 5223409
4 Clackson, T. and Wells, J. A. (1994) *Trends Biotechnol.* 12, 173–183
5 Lane, D. P. and Stephan, C. W. (1993) *Curr. Opin. Immunol.* 5, 268–271
6 Cunningham, B. C., Lowe, D. G., Li, B., Bennett, B. D. and Wells, J. A. (1994) *EMBO J.* 13, 2508–2515
7 Ladner, R. C. in *Phage Display of Peptides and Protein Domains: A Laboratory Manual* (Kay, B., Winter, J. and McCafferty, J., eds), Academic Press (in press)
8 Creighton, T. E. and Charles, I. G. (1987) *Cold Spring Harbor Symp. Quant. Biol.* 52, 511–519
9 Brendt, K. D., Guntert, P. and Wuthrich, K. (1993) *J. Mol. Biol.* 234, 735–750
10 Hynes, T. R., Randal, M., Kennedy, L. A., Eigenbrot, C. and Kossiakoff, A. A. (1990) *Biochemistry* 29, 10018–10022
11 de Vos, A. M. *et al.* (1992) *Biochemistry* 31, 270–279
12 Balass, M., Heldman, Y., Cabilly, S., Givol, D., Katchalski-Katzir, E. and Fuchs, S. (1993) *Proc. Natl Acad. Sci. USA* 90, 10638–10642
13 Goudon, R. J., Doyle, M. V., Kaufman, S. E. and Rosenberg, S. (1994) *Proc. Natl Acad. Sci. USA* 91, 7129–7133
14 Cwirla, S. E., Peters, E. A., Barrett, R. W. and Dower, W. J. (1990) *Proc. Natl Acad. Sci. USA* 87, 6378–6382
15 Pinilla, C., Appel, J. A. and Houghten, R. A. (1993) *Gene* 128, 71–76
16 McLafferty, M. A., Kent, R. B., Ladner, R. C. and Markland, W. (1993) *Gene* 128, 29–36
17 Hoess, R. H., Mack, A. J., Walton, H. and Reilly, T. M. (1994) *J. Immunol.* 153, 724–729
18 Leatherbarrow, R. J. and Salcinski, H. I. (1991) *Biochemistry* 30, 10717–10721
19 Koivunen, E., Wang, B. and Ruoslahti, E. (1994) *J. Cell Biol.* 124, 373–380
20 Koivunen, E., Wang, B. and Ruoslahti, E. (1995) *Bio/Technology* 13, 265–270
21 Dennis, M. S. and Lazarus, R. A. (1994) *J. Biol. Chem.* 269, 22129–22136
22 Dennis, M. S. and Lazarus, R. A. (1994) *J. Biol. Chem.* 269, 22137–22144
23 Clackson, T. and Wells, J. A. (1995) *Science* 267, 383–386
24 Feng, S., Chen, J. K., Yu, H., Simon, J. A. and Schreiber, S. L. (1994) *Science* 266, 1241–1247
25 Scott, J. K. and Smith, G. P. (1990) *Science* 249, 386–390
26 Devlin, J. J., Panganiban, L. C. and Devlin, P. E. (1990) *Science* 249, 404–406
27 Kay, B. K., Adey, N. B., He, Y-S., Manfredi, J. P., Mataragnon, A. H. and Fowlkes, D. M. (1993) *Gene* 128, 59–65
28 Roberts, B. L. *et al.* (1992) *Proc. Natl Acad. Sci. USA* 89, 2429–2433
29 Smith, G. P., Schultz, D. A. and Ladbury, J. E. (1993) *Gene* 128, 37–42
30 Folgori, A. *et al.* (1994) *EMBO J.* 13, 2236–2243
31 Meola, A. *et al.* (1995) *J. Immunol.* 154, 3162–3172

Progress in anti-HIV structure-based drug design

Michael J. Gait and Jonathan Karn

The course of drug development for the treatment of HIV-1 infection and AIDS is being revolutionized by high-resolution structures of essential viral proteins. We survey the impact on drug design of the recently elucidated structural knowledge of two essential enzymes, reverse transcriptase and protease, and three new targets, the viral integrase and the gene regulatory protein–RNA interactions, Tat–TAR and Rev–RRE.

The speed of drug development for the treatment of human immunodeficiency virus (HIV) infection is unprecedented. In the 12 years since the discovery of the virus, four antiviral drugs have been licensed – three of these within the past two years – and scores more are undergoing clinical trials. However, despite this enormous effort, effective regimens for the prevention of the progression to clinical symptoms of acquired immune deficiency syndrome (AIDS) and the treatment of AIDS patients have not yet been developed. Typically, antiviral drugs produce a marked decrease in circulating HIV and an increase in $CD4^+$ T lymphocytes as a result of their anti-HIV properties. Unfortunately, these beneficial effects are short-lived, owing to the rapid emergence of drug-resistant strains.

Current strategies for 'trapping' the virus are based on finding combinations of drugs that are more effective than monotherapy. In theory, combinations of drugs aimed at multiple targets could reduce virus replication sufficiently to make the emergence of resistant variants highly unlikely. Alternatively, it may

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CLINICAL PERSPECTIVES

Challenges in the therapy of HIV infection

Robert Yarchoan, Hiroaki Mitsuya and Samuel Broder

Drugs that inhibit human immunodeficiency virus (HIV) replication have been shown to have clinical utility in patients with HIV infection. However, the immunological improvement induced by available anti-HIV therapies in patients with acquired immune deficiency syndrome (AIDS) is incomplete and transient. Explanations for this may include immunological barriers to complete reconstitution, low therapeutic indices of the available drugs, and the development of viral resistance. An understanding of these processes, as discussed here by Robert Yarchoan and colleagues, *may provide important leads for the development of improved therapy for AIDS.*

Since the discovery of human immunodeficiency virus (HIV) as the causative agent of AIDS, a number of compounds have been identified as having *in vitro* antiretroviral activity[1]. To date, the most clinical information exists for a class of compounds called dideoxynucleosides[2–5]. Three of these compounds, zidovudine, 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC) have undergone controlled multicenter testing and are approved in the USA and elsewhere for the treatment of HIV infection.

With the available therapies, however, the immunological improvement attained is only partial and transient. Patients with advanced HIV infection who are administered zidovudine, for example, generally have $CD4^+$ T-cell count increases of only 30–60 cells mm^{-3} by week 6 to 10, after which they begin to decline[3,6]. Why does this occur? One reason is that the available drugs all have low therapeutic indices; at doses that are tolerable for long-term use, they induce only partial suppression of HIV replication. Another reason is the outgrowth of resistant HIV strains, although the relationship of this phenomenon to late falls in numbers of $CD4^+$ cells has not yet been formally proven. A third contributing factor is the toxicity of zidovudine and other drugs for immune cells. This is probably the reason that patients administered very high doses of zidovudine have an even more transient $CD4^+$ cell elevation than those receiving lower doses[6]. These concerns all indicate that the need for new anti-HIV strategies is as urgent as ever, and this issue will be discussed below.

An unresolved question is whether inhibition of HIV replication in patients with advanced HIV infection will ever be sufficient in itself to induce complete and prolonged immunoreconstitution. The ability of HIV to destroy $CD4^+$ lymphocytes *in vitro* initially suggested that the fall in CD4 cell counts in HIV-infected patients occurred through their direct destruction *in vivo*. However, the observation that relatively few (less than 1 in 10000) CD4 cells in the peripheral blood of patients contain actively replicating HIV as measured by *in situ* hybridization[7], suggested that indirect mechanisms may be involved, and a number of potential mechanisms have since been defined[8] (Table I).

For example, HIV binding to CD4 cells may cause apoptosis, perhaps in part mediated by binding of the viral-associated major histocompatibility complex molecule, HLA DR, to the T-cell receptor or conceivably by HIV acting as a superantigen[9,10]. Cells to which HIV gp120 binds may also be killed by anti-gp120 antibodies, by antibody-dependent cellular cytotoxicity, or by cytotoxic T cells[11]. Moreover, processes such as T-cell dysfunction or death caused by anti-CD4, anti-HLA DR antibodies, or other autoantibodies might continue the process of immune destruction even in the face of complete viral suppression[12]. Also, there is evidence that gp120 of HIV, or suppressive factors and cytokines released in HIV-infected patients, may impair the maturation and stimulation of immune elements[8,13,14]. Finally, there is some evidence that dysfunction of dendritic cells (or other antigen-presenting cells) as a result of HIV infection might contribute to immune dysfunction[15,16]. It should be noted that recent findings have indicated that more than 1 in 100 of the $CD4^+$ cells in the lymph nodes of AIDS patients

TABLE I. Some possible indirect mechanisms of immune suppression in HIV infection

Destruction of cells binding HIV or gp120 by anti-HIV antibodies, cytotoxic T-cell responses, antibody-dependent cellular cytotoxicity
Abnormal T-cell maturation induced by thymic damage or thymic HIV infection
Destruction of the architecture of lymph nodes and other lymphoid organs
Autoantibodies to antigens on immune cells (for example to HLA class II molecules or CD4)
CD4 T-cell dysfunction caused by the binding of gp120 to CD4
Overproduction of suppressive factors or toxic cytokines
Programmed cell death (apoptosis) induced by the binding of gp120 to CD4, possibly in conjunction with HLA DR incorporated into HIV
Effects of circulating immune complexes
Suppression of bone marrow stem cells and other progenitor cells by direct HIV infection or by destruction of the bone marrow microenvironment
Suppressive effects of opportunistic infections (for example cytomegalovirus)
Perturbation of T-cell repertoire induced by virally-encoded superantigens
Dysfunction of dendritic cells and other antigen-presenting cells

HIV, human immunodeficiency virus; HLA DR, a component of the major histocompatibility complex.

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D. J. Back

Pharmacological Issues Relating to Viral Resistance

Summary: Anti-HIV drug regimens can fail for a number of reasons including virological resistance, difficulties of adherence and poor tolerability. However, this brief review focuses on the development of "pharmacological" resistance as an area of great importance in drug failure. For nucleoside reverse transcriptase inhibitors (NRTIs) this is related to the possible down-regulation of the intracellular phosphorylation of an NRTI with time. For protease inhibitors the concern is cells expressing transmembrane energy-dependent transporters (such as p-glycoprotein, p-gp; or multi-drug resistance protein, MRP) which efflux drug (particularly protease inhibitors) out of the cell so that intracellular concentrations of drug are insufficient for antiviral effect.

Introduction

The ultimate goal of antiretroviral therapy in HIV-positive patients is the total eradication of the virus. Current treatment strategies are aimed at maximizing suppression of viral replication using a combination of nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). The general approach to the treatment of patients has changed significantly over the past 2 years. This follows a number of recent advances, including a better understanding of HIV pathogenesis, the development of more sensitive and accurate quantification of HIV-RNA in plasma and the availability of newer antiretroviral agents. The use of PIs in combination with reverse transcriptase inhibitors has been associated with sustained suppression of viral replication, reduced morbidity and prolonged life in patients with HIV infection [1, 2]. However, alongside the encouraging progress in treatment, it is important to recognize that even a low level of replication may result in viral diversity and hence resistance to antiretrovirals. Although drug regimens may fail for a number of reasons (including virological resistance, poor tolerability, difficulties with adherence and persistent virus in sanctuary sites), the development of cellular (pharmacological) resistance related to decreased intracellular phosphorylation of NRTIs over time is a major concern. Another possible mechanism of cellular resistance is altered influx of drug into cells or increased efflux of drug from cells.

Intracellular Phosphorylation

All the NRTIs (zidovudine, ZDV; lamivudine, 3TC; didanosine, ddI; zalcitabine, ddC; stavudine, d4T; abacavir, ABC) require intracellular phosphorylation to the active triphosphate form, after which they are potent terminators of the growing viral DNA. One reason for the relatively poor suppression of viral replication may well be inefficient metabolic activation [3]. A fall in the amount of any activating kinases may result in subtherapeutic levels of intracellular dideoxynucleoside triphosphate (ddNTP) and decreased efficacy of antiretroviral therapy. It has been reported that *in vitro* cell lines showing ZDV resistance have decreased thymidine kinase and deoxythymidylate kinase activity [4]. Although reduced thymidine kinase activity has been demonstrated in peripheral blood mononuclear cells (PBMCs) isolated from HIV-positive individuals compared with HIV-negative controls, the *in vivo* data are much less clear.

The ALTIS trial demonstrated a difference in response between ZDV-naive and ZDV-experienced patients after antiretroviral therapy with d4T plus 3TC [5]. The drop in HIV plasma RNA in patients who commenced therapy with d4T plus 3TC was greater in those who had not previously received ZDV alone or in combination (0.66 $\log_{10}$ versus 1.66 $\log_{10}$ drop). Intracellular levels of d4T triphosphate and 3TC triphosphate were determined by mass spectrometry; although there was wide variability, the results demonstrated reduced d4T triphosphate and 3TC triphosphate levels in ZDV-experienced patients who had not responded to the dual combination. It was postulated that prior therapy with ZDV hampered subsequent phosphorylation of d4T and, to a lesser extent, 3TC. The reduced d4T activation was thought to be the result of decreased thymidine kinase activity *in vivo*.

However, more recent data from a study investigating ZDV activation in a heterogeneous population of HIV-positive patients showed no decrease in phosphorylation over time (Figure 1) [6]. Additionally, there was no difference between activation in ZDV-naive and ZDV-experienced patients. These results demonstrate that if a decrease in thymidine kinase expression occurs during long-term therapy, ZDV phosphorylation is unaffected by this decrease. Since thymidine kinase has a 600-fold higher affinity for ZDV than d4T [7], a decrease in thymidine kinase activity may only affect d4T activation because ZDV is a significantly better substrate for this enzyme. As drug efficacy is determined by the competition between ddNTP and deoxynucleoside triphosphate (dNTP), the efficacy of d4T and 3TC will be dependent on the ratio of ddNTP to dNTP. Furthermore, recent additional data

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PROTOCOL

In Vitro Assessment of Compounds for Anti-HIV Activity

Clive Patience, John Moore, and Mark Boyd*

Abstract

Human immunodeficiency virus types 1 and 2 (HIV-1, -2), the etiological agents of AIDS, are retroviruses that replicate in CD4+ T-lymphocytes, monocytes, and macrophages. Early anti-HIV therapies were directed at the step in the virus life-cycle that was considered the most readily blocked: the transcription of viral RNA into its DNA copy by the viral enzyme reverse transcriptase (RT). Unfortunately, to date, patient therapies have been relatively unsuccessful and hampered by toxicological problems. This has promoted further research into inhibitors of HIV replication, which may act at alternative stages in the viral replicative cycle as well as more effective RT inhibitors. In order to facilitate such research, simple and accurate in vitro assays are highly desirable. Here we describe such assays that measure components of the HIV replicative cycle and are suitable for use within antiviral experiments.

Index Entries: Antiviral; colorimetric; cytotoxicity; ELISA; human immunodeficiency virus (HIV); reverse transcriptase.

1. Introduction

Since the identification of HIV (human immunodeficiency virus) as the retrovirus responsible for AIDS (acquired immunodeficiency syndrome), huge efforts have been made to identify compounds possessing antiviral activity. Many assay systems are available to measure various stages of the viral life-cycle. These are very convenient for assessing the antiviral activity of compounds. The assay systems often use T-cell lines expressing CD4 on their surface, since this molecule is an essential com-

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Urokinase-type plasminogen activators include urokinase (UK) in both low and high molecular weight forms. High molecular weight UK (HMW-UK, MW of 53 kDa) is a disulfide-linked dimer having a heavy (B) chain (amino acids 159-411) and a light (A) chain (amino acids 1-158). UK is a naturally occurring serine protease which is highly specific for plasminogen, and is thus an effective fibrinolytic agent. UK is well tolerated when injected intravenously, e.g., for thrombolytic therapy, at bolus dosages as high as 20 mg. Mathey et al., Am. J. Cardiol., 55:878 (1985).

Low molecular weight UK (LMW-UK) includes the entire B chain of UK plus a small portion of the A chain connected by a disulfide bond, and has a MW of about 33 kDA when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis. LMW-UK is missing the UK receptor binding domain as described in Appella et al., J. Biol. Chem., 262:4437 (1993).

Summary of the Invention

The invention is based on the discovery that the major portion of the activation site loop of plasminogen is highly homologous, both in amino acid sequence and in three-dimensional structure, to the highly conserved sequence GPGR (SEQ ID NO:1) in the tip of the PND or V3 loop of the HIV-1 envelope protein gp120.

Furthermore, it was discovered that although urokinase-type plasminogen activators (u-PAs) are highly restricted enzymes whose principal substrate is plasminogen, these enzymes also inhibit HIV-1 infectivity, i.e., inhibit the infection of a cell by HIV-1, by cleaving the tip of the V3 loop immediately adjacent and downstream of the Arg residue (R) in the sequence GPGR (SEQ ID NO:1). This finding is in contrast

found: (1) that the factor exhibited anti-HIV activity at a surprisingly low concentration (0.74 ng/mL), (2) that it was effective on both macrophage-tropic and T cell-tropic strains of HIV, and (3) that it was likely that the factor suppressed later stages than the stage of translation of viral mRNA in the HIV life cycle, in particular the stages of assembling of viral particles or budding. While ATF has a property of specifically binding to CD87 on the surface of cells, it was also found using healthy human urine urokinase, that the high molecular weight urokinase-type plasminogen activator (HMW-uPA), which had been known to include ATF moiety at an end of its molecule and to be a ligand molecule to CD87, also had anti-HIV activity. In addition, it was confirmed that ATF obtained by decomposing healthy human urine urokinase also had anti-HIV activity. Moreover, it was found that anti-CD87 antibody had an ATF-like anti-HIV activity, and that the anti-HIV activity of ATF was mediated by the same target molecule as the anti-CD87 antibody's target (i.e., CD87). These findings made it clear that it is possible to suppress reproduction of HIV by blocking CD87 by bringing CD87 on potential HIV host cells into contact with one of specifically binding ligand molecule such as ATF, HMW-uPA or fragments thereof or analogues thereto.

Thus the present invention provides an anti-HIV agent comprising as an active component a ligand molecule binding to CD87. The ligand is, for example, the high molecular weight urokinase-type plasminogen activator. Moreover, the ligand molecule may be a fragment of or an analogue to the high molecular weight urokinase-type plasminogen activator insofar as the fragment or the analogue has a specific binding affinity to CD87. Furthermore, the ligand molecule may be the amino-terminal fragment (ATF) of the high molecular weight urokinase-type plasminogen activator, as well as a fragment of or an analogue to ATF having a specific binding affinity to CD87. Other examples of the ligand molecule include an anti-CD87 antibody (monoclonal or polyclonal), as well as a fragment of or an analogue to an anti-CD87 antibody having a specific binding affinity to CD87.

The present invention also provides a pharmaceutical composition comprising, as an active component, a ligand molecule binding to CD87. Examples of such ligand molecules are as mentioned above. Among such ligand molecules, ATF and fragments thereof or analogues thereto having a specific binding affinity to CD87 are especially preferred.

pro-urokinase, the high molecular weight urokinase-type plasminogen activator, ATF and anti-CD87 antibodies as well as their fragments or analogues having ability of specifically binding to CD87, and further includes any other compound having ability of specifically binding to CD87 and that can be administered to a patient.

HIV is divided into two subtypes, HIV-1 and HIV-2. Both HIV-1 and HIV-2 are a type of virus that is released from the host by budding, and they are genetically nearly indistinguishable. They share a common life cycle and reproduce in the same manner. Therefore, there is no need for distinguishing them from each other in the context of anti-HIV drugs, and actually they are in general viewed as being equivalent in the treatment with conventional anti-HIV drugs. In the present specification, the term "HIV" includes both "HIV-1" and "HIV-2" unless otherwise mentioned.

The high molecular weight urokinase-type plasminogen activator (HMW-uPA)(Fig. 1(b); amino acids 21-178 + amino acids 179-431) is a protein consisting of two peptide chains linked by a disulfide bond. The chains, long A and B, are formed by enzymatic cleavage (with plasmin, kallikrein, cathepsin, etc.) between amino acids 178 and 179 of pro-urokinase, which is formed by removal of the N-terminal signal peptide (amino acids 1-20) from a single chain protein called prepro-urokinase (sc-uPA)(Fig. 1(a); amino acids 1-431). HMW-uPA includes an EGF-like domain, a Kringle domain and a urokinase receptor (CD87) binding domain.

HMW-uPA then is cleaved between amino acids 155 and 156 in vivo, thereby giving rise to the law molecular weight urokinase-type plasminogen activator (LMW-uPA)(Fig. 1(c); amino acids 156-178 and amino acids 179-431) and the amino-terminal fragment (ATF)(Fig. 1(d); amino acids 21-155) that has no plasminogen activator activity. Cleavage between amino acids 155 and 156 also takes place during incubation in, e.g., a phosphate buffer solution, pH 8. Thus, ATF can be produced by simple incubation of HMW-uPA in a buffer solution (25-37℃). ATF includes the EGF-like domain, the Kringle domain and the urokinase receptor (CD87) binding domain of HMW-uPA in their entirety. In the Sequence Listing, the nucleotide sequence encoding sc-uPA and its amino acid sequence are set forth as SEQ ID NO:1 and NO:2, respectively. In the sequences set forth as SEQ ID NO:1 and NO:2, amino acids 1-20 correspond to the signal

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Biochemistry

Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes

(cell migration/phorbol ester/proteolysis)

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ABSTRACT **The purified amino-terminal fragment (ATF) of human urokinase plasminogen activator (residues 1–135), which is not required for activation of plasminogen, binds with high affinity to specific plasma membrane receptors on U937 monocytes. Intact urokinase efficiently competes for ^{125}I-labeled ATF binding; 50% competition occurs with 1 nM urokinase. A large part of receptor-bound urokinase remains on the cell surface for at least 2 hr at 37°C. Differentiation of U937 monocytes into macrophage-like cells specifically increases ATF binding 10- to 20-fold. These results suggest an important role for urokinase in monocyte/macrophage biology: the native enzyme binds to the cells with the amino-terminal domain; the catalytic, carboxyl-terminal domain remains exposed on the cell surface to stimulate localized proteolysis and facilitate cell migration.**

Urokinase, one of two plasminogen activators, is a serine protease that activates plasminogen by converting it to plasmin. Consequently, urokinase can regulate a variety of events that require extracellular proteolysis, such as cell migration, tissue remodeling, and involution (1, 2). In tumor cells, cell migration may be essential to manifest the malignant phenotypes of invasiveness and metastasis (3). In agreement with this model, many transformed cell lines secrete relatively large amounts of urokinase (reviewed in ref. 4).

The physiological function of monocytes requires cell migration. These cells must leave the blood stream, pass through the endothelial wall and basement membrane, enter the neighboring tissue, differentiate into macrophages, and participate in phagocytosis (5). Plasminogen activators may facilitate the early stages of this cascade by contributing to the dissolution of basement membrane and the breaking of intercellular bridges (1, 6). This possibility has been strengthened by the recent report of Vassalli *et al.* (7), who showed that urokinase binds specifically to freshly isolated blood monocytes and to the U937 monocyte line.

Urokinase (8) is synthesized as a single-chain prepropolypeptide (9), secreted as an inactive single-chain prourokinase zymogen (411 residues) (10–12), and activated by proteolysis, which removes lysine-158 (10) to generate a two-chain urokinase molecule (residues 1–157 and 159–411; M_r = 50,000) (13, 14). This form of active urokinase is referred to as high molecular weight (HMW) urokinase. Another active form of the enzyme (the M_r 33,000 urokinase) contains only the carboxyl-terminal two-thirds of HMW urokinase (residues 136–157 and 159–411) (13). Thus, the amino-terminal portion of HMW urokinase (residues 1–135) is not required for catalytic activity.

We have prepared specific fragments of urokinase (the amino-terminal peptide, which lacks proteolytic activity, and the carboxyl-terminal peptide, which contains the catalytic domain) and have examined the binding of these fragments to urokinase receptors. We report that (*i*) the purified amino-terminal fragment of urokinase (ATF; residues 1–135) is totally sufficient to bind specifically to the urokinase receptor on U937 monocytes; (*ii*) the receptor-bound ligand remains on the cell surface, possibly to stimulate localized proteolysis through the catalytic, carboxyl-terminal domain; and (*iii*) differentiation of the monocytes into macrophage-like cells results in a 10- to 20-fold increase in ATF binding.

MATERIALS AND METHODS

Materials. Human urinary urokinase was purified to homogeneity at Lepetit Spa Laboratories (specific activity, 120,000 international units/mg). Epidermal growth factor was purified from male mouse submaxillary glands similarly to the procedure described (15). ^{125}I-labeled insulin was purchased from New England Nuclear. Phorbol 12-myristate 13-acetate (PMA; LC Service, Woburn, MA) was dissolved at 1 mg/ml in dimethyl sulfoxide and diluted to working concentrations with RPMI 1640 medium (GIBCO) containing 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD). Purified tissue plasminogen activator (80,000 international units/mg) was the gift of Keith Marotti (Upjohn).

Purification of ATF. Purified HMW urokinase (30 mg in 1 ml) was incubated for 8 hr in 50 mM sodium phosphate buffer, pH 8/0.2 M NaCl. Reaction products were separated by gel filtration at a flow rate of 3 ml/hr on a column (1.5 × 100 cm) of Sephadex G-100 (superfine) equilibrated with 50 mM sodium phosphate buffer, pH 8.0/0.2 M NaCl. Fractions containing ATF were pooled and directly subjected to ion-exchange chromatography using a fast protein liquid chromatography (FPLC) apparatus (Pharmacia) and a Mono S HR5/5 column equilibrated with 50 mM sodium acetate buffer, pH 4.8. A sodium chloride gradient (0–1.0 M in 35 min) was used to elute bound proteins. FPLC purified ATF to homogeneity with an overall recovery of 1.4 mg.

Radioiodination. ATF and HMW urokinase were radioiodinated as described (16) using 1 nmol of carrier-free Na^{125}I (Amersham), 2 nmol of chloramine-T, and 0.1 nmol of

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Abbreviations: HMW urokinase, high molecular weight urokinase; M_r 33,000 urokinase, active urokinase containing residues 136–157 and 159–411; ATF, amino-terminal fragment of urokinase containing residues 1–135 (M_r, 17,000); PMA, phorbol 12-myristate 13-acetate; FPLC, fast protein liquid chromatography.

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protein. Radiolabeled proteins were purified by gel filtration and stored as described (17). The specific activity of the labeled proteins ranged from 50 to 120 μCi/μg (1 Ci = 37 GBq).

Receptor Binding. U937 monocytes (18) (grown in RPMI 1640 medium/10% heat-inactivated fetal bovine serum to a density of 10^6 cells per ml) were collected by centrifugation and suspended (5×10^6 cells per ml) in serum-free RPMI 1640. After incubation (1 hr at 37°C in 5% CO_2/95% air), 1-ml portions of the suspension were distributed to 1.5-ml Eppendorf tubes and the tubes were centrifuged at $1000 \times g$ for 5 min. The cell pellet was suspended in 0.2 ml of binding buffer (RPMI 1640/50 mM Hepes, pH 7.4, containing bovine serum albumin at 1 mg/ml) containing ligand and rocked gently for 1 hr at 23°C (see figure legends for details of individual experiments). Cell-associated radioactivity was determined as described (19). Nonspecific binding was measured in the presence of 0.5 nM unlabeled HMW urokinase or ATF. Ligands were quantitated by amino acid analysis after hydrolyzing aliquots of stock solutions in 6 M HCl (24 hr, 110°C).

Phorbol Ester-Induced Differentiation. U937 monocytes (0.5×10^6/ml) were differentiated to macrophage-like cells by incubation in medium containing 150 nM PMA (20). A 4-day treatment resulted in a totally adherent cell population that actively phagocytized latex beads (21). About 30% of the differentiated cells internalized latex during a 1-hr incubation at 37°C. About 90% of the differentiated cells were surrounded by a crown of beads. Control cells did not show these effects. Binding of ^{125}I-labeled ATF or ^{125}I-labeled insulin to untreated and PMA-treated cells was carried out as described above, except that 0.5×10^6 cells were used per tube and cells (control and differentiated) were incubated with labeled ligand at 4°C for 150 min. Adherent cells were collected with a rubber policeman and dispersed to a single-cell suspension by repeated pipetting.

RESULTS

Preparation of Urokinase Fragments. Incubation of HMW urokinase at pH 8 results in the cleavage of native enzyme at position 135 and production of specific amino- and carboxyl-terminal fragments (Fig. 1*A Inset*). These reaction products were separated by gel filtration (Fig. 1*A*). The column fractions were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (22) and assayed for enzymatic activity (23). Peaks 1 and 2 from the gel filtration column contained,

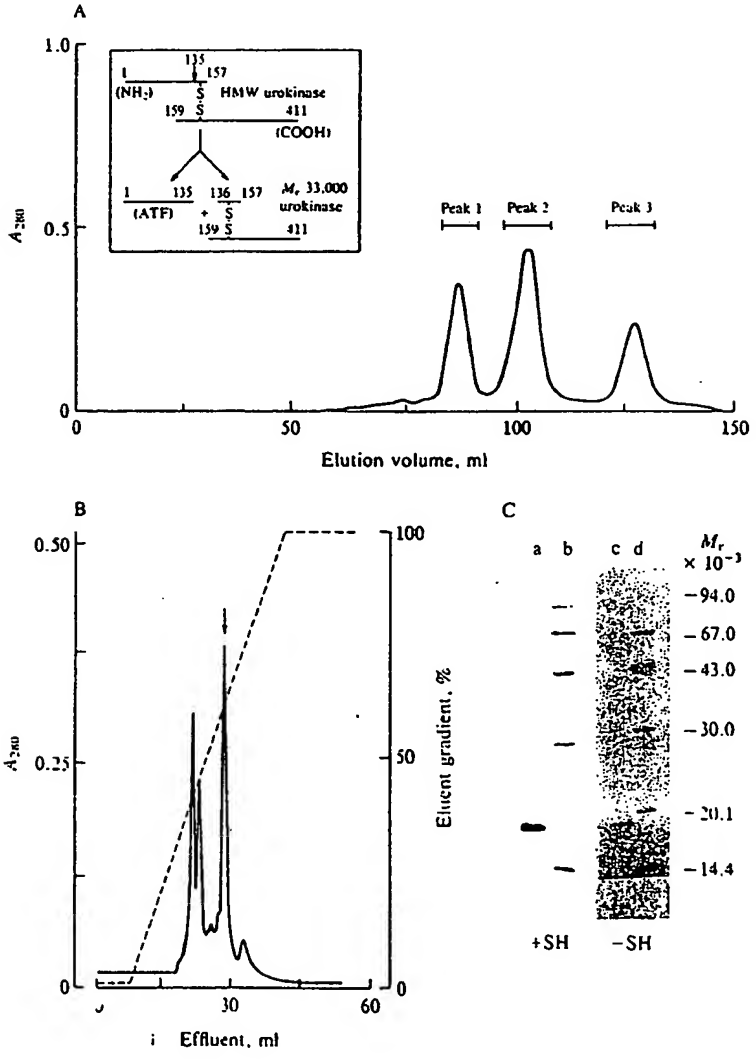

FIG. 1. Purification of ATF. (*A*) Human urokinase was cleaved by incubation at pH 8, and the reaction products were gel-filtered on Sephadex G-100. (*Inset*) Products obtained from the pH 8 incubation. (*B*) Profile obtained by FPLC of the material in peak 3 from the G-100 column. The arrow indicates the fraction containing pure ATF as determined by 12.5% NaDodSO$_4$/polyacrylamide gel electrophoresis (22) followed by staining with Coomassie blue (*C*). Lanes: a and c, migration of purified ATF; b and d, molecular weight standards determined in the presence (lanes a and b) or absence (lanes c and d) of 2-mercaptoethanol (22). Molecular weight standards were from Pharmacia.

respectively. HMW urokinase and a fully active M_r 33,000 urokinase (170,000 international units/mg) corresponding to the carboxyl-terminal urokinase fragment. Peak 3 contained two peptides (M_r, 17,000) that lacked enzymatic activity (data not shown).

The major component in peak 3 was purified to homogeneity by FPLC (Fig. 1*B*). The FPLC fraction indicated by the arrow (Fig. 1*B*) contained a single M_r 17,000 peptide as determined by $NaDodSO_4$ gel electrophoresis under both reducing and nonreducing conditions (Fig. 1*C*). The amino acid composition of this peptide (Table 1) was in complete agreement with that predicted from the primary sequence of residues 1–135 in human urokinase (14). In addition, greater than 90% of the M_r 17,000 peptide was immunoprecipitated by a monoclonal antibody specific for the amino-terminal region of HMW urokinase (data not shown). The carboxyl-terminal fragment of urokinase (M_r 33,000 urokinase) was obtained from peak 2 of the gel filtration column and used without further purification.

Specific Binding of ATF to U937 Monocytes. Competition assays were used to examine the domains of urokinase responsible for receptor binding in U937 cells. The results of an experiment in which radioiodinated HMW urokinase was bound to cells and the two urokinase fragments, ATF and the M_r 33,000 urokinase, were used as unlabeled competitors, are shown in Fig. 2*A*. In agreement with the results of Vassalli *et al.* (7), M_r 33,000 urokinase does not compete for ^{125}I-labeled urokinase binding. In addition, we find that ATF binds to the urokinase receptor with an affinity similar to or slightly greater than that of the native enzyme: 50% of maximal competition occurs with 0.2 nM ATF and 0.5 nM HMW urokinase.

The complementary competition assay, in which ^{125}I-labeled ATF was used as the probe and ATF or HMW urokinase was used as unlabeled competitor, was also carried out. [Binding of ^{125}I-labeled ATF to U937 cells is time- and temperature-dependent (Fig. 2*B Inset*): 50% of apparent steady-state binding is attained within 5 min at 37°C, 7 min at 23°C, and 20 min at 4°C.] The results show (Fig. 2*B*), again, that the relative affinity of ATF for the urokinase receptor (50% competition at 0.4 nM) is similar to, or slightly greater than, that of HMW urokinase (50% competition at 1 nM). Taken together, these data show that ATF contains all the binding determinants present in native urokinase; the catalytic carboxyl-terminal portion of the enzyme has no role in receptor binding. Note that the affinities of both ATF and HMW urokinase are within the range of plasma urokinase concentration (24).

Table 1. Amino acid composition of purified ATF

Amino acid	Residues per mol protein	
	Found	Expected
Asx	17.4	18
Thr	5.7	6
Ser	8.2	8
Glx	12.2	11
Pro	7.1	7
Gly	12.5	12
Ala	5.4	5
Cys	12.3	12
Val	8.6	7
Met	1.8	2
Ile	2.0	2
Leu	8.7	9
Tyr	5.3	6
Phe	2.9	3
His	7.1	8
Lys	8.3	9
Arg	6.4	7
Trp	ND	3

ATF (5 μg) was hydrolyzed *in vacuo* with constant-boiling HCl (24 hr at 110°C) containing a trace amount of phenol. Analysis was performed using ninhydrin for detection. Half-cystine content (Cys) was determined after performic acid oxidation. Valine and isoleucine content was determined after 72 hr of hydrolysis. Tryptophan content was not determined (ND). The composition was determined by comparison with the known primary sequence of human urokinase (14).

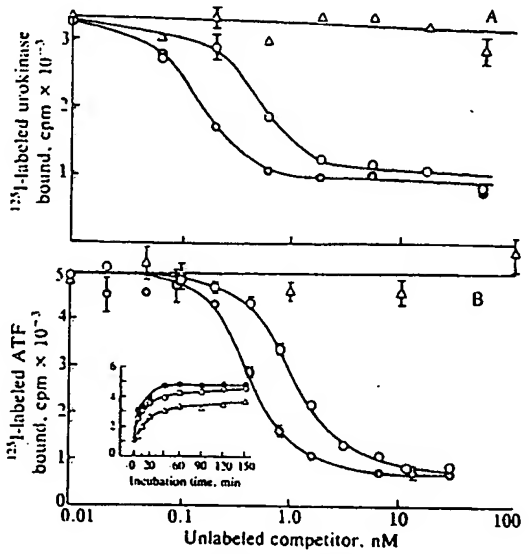

FIG. 2. Specific binding of ATF to U937 cells. Binding of ATF and HMW urokinase to U937 monocytes was as determined by radioreceptor competition assays. (*A*) Competition between ^{125}I-labeled HMW urokinase (1.5×10^5 cpm per tube) and unlabeled ATF (o), the M_r 33,000 urokinase (Δ), or HMW urokinase (O) for binding to the urokinase receptor on U937 cells. (*B*) Results of an experiment in which ^{125}I-labeled ATF was used as the probe (3×10^4 cpm per tube) and HMW urokinase (O), ATF (o), or epidermal growth factor (Δ) was used as unlabeled competitor. Data are shown as means of duplicate determinations; ranges (when observed) are indicated by error bars. (*Inset*) Time-dependent binding of ^{125}I-labeled ATF to U937 cells at 4°C (Δ), 23°C (O), and 37°C (o).

Although ATF is structurally related to epidermal growth factor (14), this protein did not compete with ^{125}I-labeled ATF for receptor binding (Fig. 2*B*). In agreement with the results of Vassalli *et al.* (7), insulin, tissue plasminogen activator, thrombin, and coagulation factors IX and X do not compete for ^{125}I-labeled ATF binding (not shown).

To examine the fate of ATF subsequent to receptor binding, ^{125}I-labeled ATF was incubated with U937 cells at 4°C to prepare a ligand–receptor complex, the unbound label was removed, and the cells were then incubated (2 hr at 37°C) in fresh medium without additional ligand. Under conditions similar to these, the majority of insulin–receptor complexes on U937 cells are internalized, the ligand is degraded, and trichloroacetic acid-soluble products are released into the medium during the 37°C incubation (25). Interestingly, cell-associated ^{125}I-labeled ATF was degraded poorly by the U937 cells; only 20% of the cell-associated radioactivity appeared in the medium as trichloroacetic acid-soluble products during incubation for 2 hr at 37°C (Fig. 3).

^{125}I-labeled ATF–receptor complexes, or ^{125}I-labeled HMW urokinase–receptor complexes, were also treated with trypsin to determine the degree of ligand internalization (Table 2). Consistent with many ligand–receptor interactions,

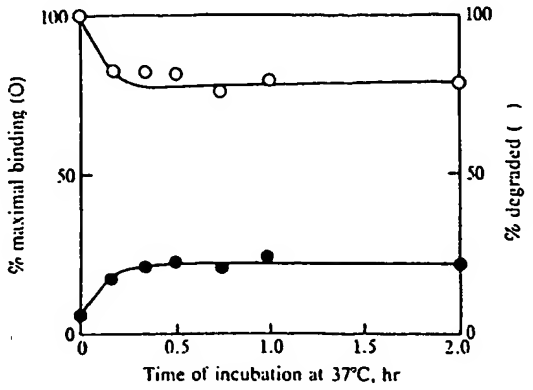

FIG. 3. Fate of receptor-bound ATF. U937 cells were incubated with ^{125}I-labeled ATF (0.3×10^5 cpm) for 2.5 hr at 4°C to form cell-surface ligand–receptor complexes. The cells were washed twice (by repeated suspension in 1-ml portions of binding buffer followed by mild centrifugation) to remove unbound label. These "pre-loaded" cells were suspended in binding buffer (0.2 ml) at 37°C to allow internalization and degradation of the ligand. At the indicated times, cells were centrifuged, the medium was collected, and the radioactivity (O) in the pellet was determined. Cell-mediated degradation of ligand to low molecular weight products (●) was determined by making the collected cell media 10% in trichloroacetic acid, incubating the solution at 4°C, and centrifuging the resulting suspension at $10,000 \times g$ to prepare trichloroacetic acid-soluble and -precipitable fractions. Maximal binding of ^{125}I-labeled ATF (cpm bound after the 4°C incubation) was about 800 cpm.

the radioactivity associated with the U937 monocytes after the 4°C incubation remained on the cell surface as determined by its susceptibility to treatment with trypsin. Even after incubation of the cells for 2 hr at 37°C, the majority of cell-associated ATF and HMW urokinase remained sensitive to trypsin. Taken together, the results of Fig. 3 and Table 2 indicate that most of ATF and HMW urokinase associated with U937 cells remains exposed on the plasma membrane. Note that the percentage of cell-associated radioactivity sensitive to trypsin is somewhat greater for HMW urokinase than it is for ATF. This difference may be related to the larger size and increased number of tryptic cleavage sites in HMW urokinase relative to ATF.

PMA-Induced Differentiation of U937 Monocytes Increases ATF Binding. PMA differentiates U937 monocytes into macrophage-like cells (20). We have examined the effect of differentiation on the binding of ^{125}I-labeled ATF to the urokinase receptor. These experiments were performed at 4°C, conditions in which cell-associated radioactivity is restricted to the plasma membrane (refs. 26 and 27 and Table 2). To estimate ATF binding capacity in control and PMA-treated cells, increasing amounts of unlabeled ATF were allowed to compete for ^{125}I-labeled ATF binding; the concentration of unlabeled ligand required to induce the onset of competition provides a measure of binding capacity. This method of analysis (Fig. 4*A*) shows that PMA-treated cells bind about 20-fold more ATF than their untreated counterparts. Saturation plots (28) of the binding data (Fig. 4*A Inset*) show that PMA treatment does not dramatically alter the relative affinity of the urokinase receptor (half-maximal binding is attained with 2 nM ATF in the differentiated cells), indicating that differentiation of U937 cells with PMA likely increases the number of ATF binding sites. Importantly, differentiation of the monocytes with PMA did not alter their binding of insulin (Fig. 4*B*).

We have considered the possibility that increased ATF binding to the macrophage-like cells might be a direct effect of PMA rather than a consequence of differentiation. By

Table 2. Trypsin treatment of receptor-bound ATF and urokinase

Ligand	Cell-associated radioactivity, cpm: Incubation at 4°C	Incubation at 37°C
^{125}I-labeled ATF		
Without trypsin	897	1768
With trypsin	229	663
^{125}I-labeled HMW urokinase		
Without trypsin	2800	2130
With trypsin	241	291

U937 cells were pre-loaded with ^{125}I-labeled ATF or ^{125}I-labeled HMW urokinase as described in the legend to Fig. 3 except that 10^5 cpm of each ligand was used. Washed cells were suspended in 0.5 ml of fresh binding buffer and either treated with trypsin (0.2 mg/ml for 15 min at 37°C; see ref. 26) immediately or incubated at 37°C for 2 hr prior to trypsin treatment. Controls ("without trypsin") were always treated in parallel except that the incubation for 15 min at 37°C was performed in the absence of trypsin. Trypsin-sensitive radioactivity ranged from 60 to 75% and 85 to 92% of control binding for ATF and HMW-urokinase, respectively.

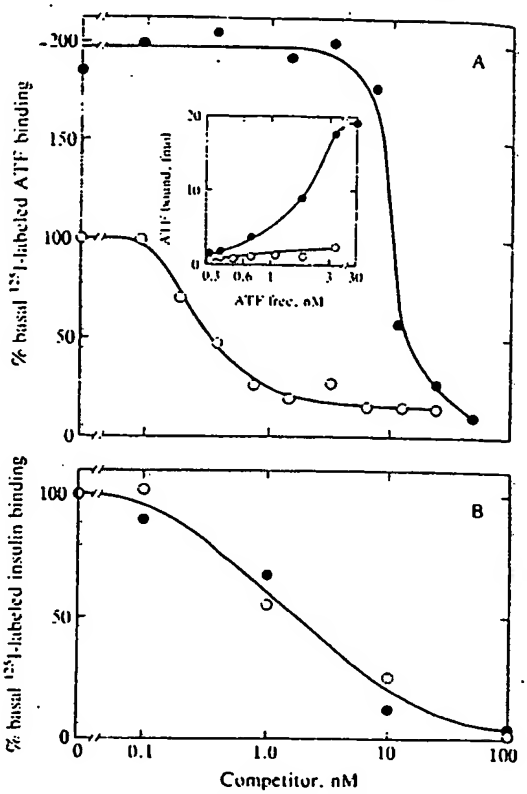

FIG. 4. PMA-induced differentiation alters urokinase binding. (*A*) ATF binding capacity in control (O) and differentiated (●) U937 cells was estimated by competition assay using ^{125}I-labeled ATF (1.5×10^5 cpm per tube) and unlabeled ATF as competitor. Results are presented as percent of basal binding (^{125}I-labeled ATF binding to control cells in the absence of competitor; 2500 cpm). Data are corrected for nonspecific binding (about 20% of total binding). (*Inset*) ATF binding data plotted as saturation curves. (*B*) Comparison of binding capacity of untreated (○) and PMA-treated (●) cells for insulin (determined as described for *A* using 8×10^4 cpm of ^{125}I-labeled insulin; basal ^{125}I-labeled insulin binding was about 800 cpm).

treating U937 cells with PMA for 24 or 48 hr, we could obtain a population of adherent macrophage-like cells as well as a nonadherent cell population that had yet to differentiate. (Note, however, that both cell populations were exposed to PMA for the same period of time.) Control studies, performed as described in Fig. 4*A*, showed that the adherent cells bound 10-fold more ATF than the nonadherent population (data not shown). Thus, the increased ATF binding described in Fig. 4 is likely a consequence of differentiation.

DISCUSSION

We have prepared specific fragments of urokinase to examine the domains responsible for receptor binding. The results show that urokinase binds to specific cell surface receptors on U937 cells solely through the amino-terminal portion of the enzyme. Interestingly, the majority of cell-associated ATF or HMW urokinase remains on the cell surface during incubation at 37°C. These results suggest a model for urokinase binding to U937 monocytes in which the amino-terminal portion of the enzyme binds to a plasma membrane receptor while the catalytic, carboxyl-terminal domain of the molecule remains exposed on the plasma membrane to stimulate extracellular proteolysis.

In vivo, the binding of urokinase to monocytes might serve to localize proteolysis and facilitate migration of these cells through the walls of blood vessels. In addition to supporting previous studies demonstrating that cell migration is plasminogen-dependent and impaired by protease inhibitors (6, 29), our model of urokinase binding lends itself to direct testing; ATF should be an inhibitor of receptor-mediated urokinase action. It is also likely that cell-surface urokinase can serve additional purposes; studies by others have indicated a role for plasminogen activator in lymphocyte activation and tumor antigenicity (30–32).

Differentiation of U937 monocytes into macrophage-like cells specifically increases ATF binding to the urokinase receptor. Other studies have shown that stimulation of peritoneal macrophages induces secretion of urokinase (33). This coordinate regulation of ligand and receptor expression emphasizes the likely role of cell-surface urokinase in the inflammatory response and agrees with the well-documented stimulation of proteolysis that accompanies inflammation (1). A detailed comparison of the monocyte and macrophage urokinase receptors is an important matter for further study.

The presence of a urokinase receptor on monocytes has implications in tumor cell biology. Although many malignant tumors and tumor cells secrete high amounts of urokinase, not all of them do (4). Some tumor cells might circumvent the need for increased urokinase synthesis by having urokinase receptors that could concentrate the enzyme on their cell surface and localize extracellular proteolysis. Should this be the case, the discrepancies in plasminogen activator levels in different tumor cells (4) might be reconciled.

Higher eukaryotes have evolved different mechanisms to partition proteins between the cell surface and the extracellular fluid. For example, differential RNA splicing can give rise to both membrane and secreted forms of IgM (34). Cells can also have membrane receptors that bind proteins present in plasma. In these receptor systems, ligand binding initiates a cellular response by transmitting a signal and also limits the response by inducing rapid internalization and degradation of the effector (26). U937 cells secrete (35) and bind urokinase, but they internalize the ligand poorly. These data suggest a novel variation of the autocrine mechanism (36): a secreted effector binds to its plasma membrane receptor and acts extracellularly while on the cell surface.

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1. Reich, E. (1978) in *Molecular Basis of Biological Degradative Processes*, eds. Berlin, R. D., Herrmann, H., Lepow, I. H. & Tanzer, J. M. (Academic, New York), pp. 155–169.
2. Dano, K., Andreasen, P. A., Grondhal-Hansen, J., Kristensen, P., Nielsen, L. S. & Skriver, L. (1985) *Adv. Cancer Res.*, in press.
3. Ossowski, L. & Reich, E. (1983) *Cell* 35, 611–619.
4. Mullins, D. E. & Rohrlich, S. T. (1983) *Biochim. Biophys. Acta* 695, 177–214.
5. Rosenstreich, D. L. (1981) in *Cellular Functions in Immunity and Inflammation*, eds. Oppenheim, J. J., Rosenstreich, D. L. & Potter, M. (Elsevier/North-Holland, New York), pp. 127–159.
6. Ossowski, L., Quigley, J. P., Kellerman, G. M. & Reich, E. (1973) *J. Exp. Med.* 138, 1056–1064.
7. Vassalli, J. D., Baccino, D. & Belin, D. (1985) *J. Cell Biol.* 100, 86–92.
8. Sobel, G. W., Mohler, S. R., Jones, N. W., Dowdy, A. B. C. & Guest, M. M. (1952) *Am. J. Physiol.* 171, 768–769.
9. Salerno, G., Verde, P., Nolli, M. L., Corti, A., Szots, H., Meo, T., Johnson, J., Bullock, S., Cassani, G. & Blasi, F. (1984) *Proc. Natl. Acad. Sci. USA* 81, 110–114.
10. Verde, P., Stoppelli, M. P., Galeffi, P., DiNocera, P. P. & Blasi, F. (1984) *Proc. Natl. Acad. Sci. USA* 81, 4727–4731.
11. Wun, T., Ossowski, L. & Reich, E. (1982) *J. Biol. Chem.* 257, 7262–7268.
12. Nielsen, L. S., Hansen, J. G., Skriver, L., Wilson, E. L., Kaltoft, K., Zeuthen, J. & Danø, K. (1982) *Biochemistry* 21, 6410–6415.
13. Günzler, W. A., Steffens, G. J., Otting, F., Buse, G. & Flohe, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* 363, 133–141.
14. Günzler, W. A., Steffens, G. J., Otting, F., Kim, S. M., Frankus, E. & Flohe, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* 363, 1155–1165.
15. Savage, C. R. & Cohen, S. (1972) *J. Biol. Chem.* 247, 7609–7611.
16. Assoian, R., Thomas, N., Kaiser, E. & Tager, H. (1982) *Proc. Natl. Acad. Sci. USA* 79, 5147–5151.
17. Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, D. M. & Sporn, M. B. (1984) *Cell* 36, 35–41.
18. Sundström, C. & Nilsson, K. (1976) *Int. J. Cancer* 17, 565–577.
19. Contrell, D. A. & Smith, K. A. (1983) *J. Exp. Med.* 158, 1895–1911.
20. Ralph, P., Williams, N., Moore, M. A. S. & Litkovsky, P. B. (1982) *Cell. Immunol.* 71, 215–223.
21. Cline, M. J. & Lehrer, R. I. (1968) *Blood* 32, 423–435.
22. Laemmli, U. K. (1970) *Nature (London)* 227, 680–685.
23. Plough, J. & Kjeldgaard, N. O. (1957) *Biochim. Biophys. Acta* 24, 278–282.
24. Wun, T. E., Schleuning, W. D. & Reich, E. (1982) *J. Biol. Chem.* 257, 3276–3283.
25. Robert, A., Grunberger, G., Carpentier, J. L., Dayer, J. M., Orci, L. & Gorden, P. (1984) *Endocrinology* 114, 247–253.
26. King, A. C. & Cuatrecasas, P. (1982) *J. Biol. Chem.* 257, 3053–3060.
27. Pastan, I. & Willingham, M. C. (1983) *Trends Biochem. Sci.* 8, 250–254.
28. Rodbard, D. (1973) in *Receptors for Reproductive Hormones*, eds. O'Malley, B. W. & Means, A. R. (Plenum, New York), pp. 289–326.
29. Ossowski, L., Quigley, J. P. & Reich, E. (1975) in *Proteases and Biological Control*, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 901–913.
30. Cohen, S. D., Israel, E., Spiess-Meier, B. & Wainberg, M. A. (1981) *J. Immunol.* 126, 1415–1420.
31. Miller, C. L., Graziano, C. J. & Lim, R. C. (1982) *J. Immunol.* 128, 2194–2200.
32. Reich, E. (1974) in *Control of Proliferation in Animal Cells*, eds. Clarkson, B. & Baserga, R. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 351–358.
33. Unkeless, J. C., Gordon, S. & Reich, E. (1974) *J. Exp. Med.* 139, 834–850.
34. Early, P., Huang, H., Davis, M., Calame, K., Bond, M., Wall, R. & Hood, L. (1980) *Cell* 20, 313–319.
35. Vassalli, J. D., Dayer, J. M., Wohlwend, A. & Belin, D. (1984) *J. Exp. Med.* 159, 1653–1668.
36. Sporn, M. B. & Todaro, G. J. (1980) *N. Engl. J. Med.* 303, 878–880.

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United States Patent [19]

Imamura et al.

[11] **Patent Number: 5,874,254**

[45] **Date of Patent: Feb. 23, 1999**

[54] **FGF-5 ANALOGOUS PROTEIN, AND PHARMACEUTICAL COMPOSITION CONTAINING THE SAME**

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[58] **Field of Search** 530/399; 435/69.4; 514/12

[56] **References Cited**

PUBLICATIONS

"The rate FGF–5 mRNA variant generated by alternative splicing encodes a novel truncated from of FGF–5" by Hattori et al.; Biochimica et Biophysica Acta 1306 (1996) pp. 31–33.

"The Human FGF–5 Oncogene Encodes a Novel Protein Related to Fibroblast Growth Factors" by Zhan et al.; Molecular and Cellular Biology, Aug. 1988, pp. 3487–3495.

"Expression of the murine fibroblast growth factor 5 gene in the adult central nervous system" by Haub et al.; Proc. Natl. Acad. Sci. USA, vol. 87, pp. 8022–8026, Oct. 1990, Neurobiology.

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[57] **ABSTRACT**

The present invention relates to a novel FGF-5 analogous protein derived from mature mRNA formed by directly binding exon 1 to exon 3 in the splicing of a gene coding for FGF-5 protein as well as to a pharmaceutical composition containing the same as an active ingredient. The pharmaceutical composition containing the novel FGF-5 analogous protein of the present invention as an active ingredient can regulate physiological functions of FGF-5, such as regulation of restoration or development of hairs on the head and body, regulation of trophic and functional regulation in the brain and nervous system, proliferation of fibroblasts, etc.

8 Claims, 3 Drawing Sheets

FIG.1

Human FGF-5 Analog

```
        10         20         30         40         50         60
atgagcttgtccttcctcctcctcttcttcagccacctgatcctcagcgcctgggct
M  S  L  S  F  L  L  L  L  F  F  S  H  L  I  L  S  A  W  A

        70         80         90        100        110        120
cacggggagaagcgtctcgcccccaaagggcaacccggacccgctgccactgataggaac
H  G  E  K  R  L  A  P  K  G  Q  P  G  P  A  A  T  D  R  N

       130        140        150        160        170        180
cctataggctccagcagcagacagagcagcagtagcgctatgtcttcctcttctgcctcc
P  I  G  S  S  S  R  Q  S  S  S  S  A  M  S  S  S  S  A  S

       190        200        210        220        230        240
tcctcccccgcagcttctctgggcagccaaggaagtggcttggagcagagcagtttccag
S  S  P  A  A  S  L  G  S  Q  G  S  G  L  E  Q  S  S  F  Q

       250        260        270        280        290        300
tggagcccctcggggcgccggaccggcagcctctactgcagagtgggcatcggtttccat
W  S  P  S  G  R  R  T  G  S  L  Y  C  R  V  G  I  G  F  H

       310        320        330        340        350        360
ctgcagatctacccggatggcaaagtcaatggatcccacgaagccaatatgttaagccaa
L  Q  I  Y  P  D  G  K  V  N  G  S  H  E  A  N  M  L  S  Q

       370
gttcacagatga
V  H  R  *
```

FIG.2

Mouse FGF-5 Analog

```
        10         20         30         40         50         60
atgagcctgtccttgctcttcctcatcttctgcagccacctgatccacagcgcttgggct
M  S  L  S  L  L  F  L  I  F  C  S  H  L  I  H  S  A  W  A

        70         80         90        100        110        120
cacggggagaagcgtctcactcccgaagggcaacccgcgcctcctaggaacccgggagac
H  G  E  K  R  L  T  P  E  G  Q  P  A  P  P  R  N  P  G  D

       130        140        150        160        170        180
tccagcggcagccggggcagaagtagcgcgacgttttcttcgtcttctgcctcctcacca
S  S  G  S  R  G  R  S  S  A  T  F  S  S  S  S  A  S  S  P

       190        200        210        220        230        240
gtcgcagcttctccgggcagccaaggaagcggctcggaacatagcagtttccagtggagc
V  A  A  S  P  G  S  Q  G  S  G  S  E  H  S  S  F  Q  W  S

       250        260        270        280        290        300
ccttcggggcgccggaccggcagcctgtactgcagagtgggcatcggtttccatctgcag
P  S  G  R  R  T  G  S  L  Y  C  R  V  G  I  G  F  H  L  Q

       310        320        330        340        350        360
atctacccggatggcaaagtcaatggctcccacgaagccagtgtgttaagccaaatttac
I  Y  P  D  G  K  V  N  G  S  H  E  A  S  V  L  S  Q  I  Y

   366
ggatga
G  *
```

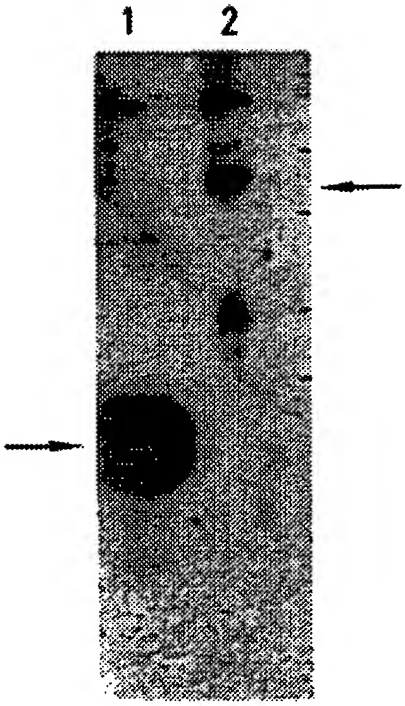

FIG.3

FGF-5 ANALOGOUS PROTEIN, AND PHARMACEUTICAL COMPOSITION CONTAINING THE SAME

FIELD OF THE INVENTION

The present invention relates to fibroblast growth factor-5 (FGF-5) analogous protein encoded by mRNA occurring by the alternative splicing of a gene encoding FGF-5 protein, as well as a pharmaceutical composition containing the same as an active ingredient, particularly a pharmaceutical composition useful for regulation of restoration or development of hairs on the head or body, trophic or functional regulation in the brain and nervous system and regulation of proliferation of fibroblasts etc.

BACKGROUND OF THE INVENTION

Conventionally, almost all components effective for promotion or suppression of restoration or development of hairs on the head or body have been obtained by screening synthetic compounds or natural substances in plants or microorganisms for those having such activity in e.g. experimental animals. However, because the trophic environments of hair roots were believed to be essential, conventional developments in hair restoration promoting agents, hair restoration suppressing agents and hair developing agents were based on improvement or suppression of topical blood circulation or environmental conditions for hair roots, but not on the mechanism of production of animal hairs.

Fibroblast growth factor-5 (FGF-5) is known to have growth promoting activity and transforming activity on fibroblasts as its physiological functions. If NIH3T3 fibroblasts are transformed with an FGF-5 gene under the control of a constant-expression vector, their growth ability is known to be altered by FGF-5. Also, FGF-5 secreted from these transformed cells into the culture significantly promotes the proliferation ability of other fibroblast BALB/c3T 3 cells. Further, an FGF-5 polypeptide expressed by *E. coli* transformed with an FGF-5 gene expression plasmid significantly promotes the proliferation ability of BALB/c3T3 as well.

The activity of FGF-5 as a neurotrophic factor is also known and expressed in skeletal muscle cells. Both FGF-5 contained in an extract of these skeletal muscle cells and FGF-5 expressed in the *E. coli* transformed with the FGF-5 gene expression plasmid are known to significantly promote the survival of cultured motor neurons. This fact strongly suggests that FGF-5 is a trophic factor of motor neurons. The expression of FGF-5 in mouse and rat brains was further found and it is believed from an experiment with brain nerve primary culture cells that FGF-5 acts as a trophic factor of cholinergic and serotonergic neurons in the brain.

The activity of FGF-5 as a neurotrophic factor came to be known as described above, and much attention is paid to a regulatory factor for the activity, but none of such regulatory factor has been found.

In the generation of the FGF-5 protein, it is known that a gene coding for the FGF-5 protein is transcribed into mRNA which in turn undergoes splicing such that exon 1, exon 2 and exon 3 are linked in this order to form a continuous chain in mature RNA. That is, its translational frame begins at the translational initiation codon ATG (coding for methionine) in exon 1, proceeds through exon 2 and ends at a termination codon in exon 3 to result in a protein composed of 268 amino acids in human or a protein of 264 amino acids in mouse (Zhan, X. et al., Mol. Cell. Biol., Vol. 8, pp. 3487–3495 (1988); Haub. O. et al., Proc. Natl. Acad. Sci., USA, Vol. 87, pp. 8022–8026 (1990)).

SUMMARY OF THE INVENTION

The object of the present invention is to provide a pharmaceutical composition useful for regulation of the physiological functions of FGF-5 e.g. regulation of hair restoration or development by direct action on the mechanism of hair production, trophic or functional regulation in the brain and nervous system, regulation of proliferation of fibroblasts, etc. as well as regulation of various physiological functions of FGF family proteins other than FGF-5 and of their gene products.

The present inventors paid attention to the presence of another FGF-5 mRNA besides the known one, and as a result of their eager research, they found that its coding protein regulates the physiological functions of FGF-5 and further that this protein acts on the fundamental mechanism of hair production to bring about regulation of hair restoration or development and trophic or functional regulation in the brain nerve system, and the present inventors arrived at completion of the present invention as a pharmaceutical composition containing the same.

That is, the present invention relates to a protein defined by splicing not known in the splicing step of generating mature mRNA (referred to as "post-translational alternative splicing of FGF-5 gene") as well as to a pharmaceutical composition containing the same.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the amino acid sequence (SEQ ID No:2) of human FGF-5 analogue (human FGF-5S) protein and a DNA sequence (SEQ ID No:1) coding for said protein.

FIG. 2 shows the amino acid sequence (SEQ ID No:4) of mouse FGF-5 analogue (mouse FGF-5S) protein and a DNA sequence (SEQ ID No:3) coding for said protein.

FIG. 3 shows a photograph of mouse FGF-5S protein and human FGF protein in SDS-polyacrylamide gel electrophoresis.

DETAILED DESCRIPTION OF THE INVENTION

Hereinafter, the present invention is described in detail.

The novel FGF-5 protein of the present invention (referred to hereinafter as FGF-5 analogous protein) is derived from mature mRNA formed by directly binding exon 1 to exon 3 in the splicing of a gene coding for FGF-5 protein.

The FGF-5 analogous protein is identical with the known FGF-5 protein in the amino acid sequence (to the 118-position in human or to the 116-position in mouse) encoded by exon 1, but is different in the amino acid sequence beginning at the 119-position in human or the 117-position in mouse because exon 1 is linked directly to exon 3, and there appears a termination codon just after coding for amino acid 123 in human or amino acid 121 in mouse. Therefore, the FGF-5 analogous protein has a novel peptide-linked structure while having a partial structure of the FGF-5 protein.

The FGF-5 analogous protein of the present invention is specifically a protein having an amino acid sequence substantially shown in FIG. 1 (SEQ. ID No:2) for human and in FIG. 2 (SEQ. ID No:4) for mouse, and the former is encoded by the DNA sequence as shown in FIG. 1 (SEQ. ID No:1) and the latter by that in FIG. 2 (SEQ. ID No:3) . The term "substantially" means that a part of said amino acid sequence may have undergone addition, deletion, replacement and modification unless its functions are lost.

The FGF-5 analogous protein referred to in the present invention includes not only a protein primarily defined by the cDNA shown in the Sequence Listing, but also a protein from which a peptide sequence called signal peptide present at the N-terminal when secreted from cells has been cleaved off. That is, the FGF-5 analogous protein contained as the active ingredient in the form of a recombinant etc. in the pharmaceutical composition of the present invention will not change its usefulness even if produced in the form of a signal peptide-free protein from the start.

Hereinafter, the method of preparing the FGF-5 analogous protein is specifically described.

RNA is extracted from animal brain tissues, and its reverse transcription is carried out using a random hexaoligonucleotide as a primer, followed by amplification by PCR techniques. If an oligonucleotide capable of amplifying the open reading frame in FGF-5 is used as the primer in reverse transcription, a DNA fragment corresponding in size to the FGF-5 protein analog of the present invention will be formed together with a DNA fragment corresponding in size to the known FGF-5 protein. The former is separated from the latter in gel electrophoresis, then cut off from the gel and inserted into a multicloning site in a cloning vector to give a plasmid.

The DNA may be integrated into any plasmid if it can be replicated and retained in a host. Examples are *E. coli*-derived pBR322, pUC18, and their derivatives such as pET-3c etc.

The DNA can be integrated into the plasmid as described e.g. by T. Maniatis et al. in Molecular Cloning, Cold Spring Harbor Laboratory, p. 239 (1982).

An expression vector can be obtained by linking the cloned gene to a region downstream of a promoter in a vector suitable for expression. Examples of such vectors are the above-described *E. coli*-derived plasmids (pBR322, pBR325, pUC12, pUC13, pET-3), *Bacillus subtilis*-derived plasmids (pUB110, pTP5, pC194), yeast-derived plasmids (pSH19, pSH15), bacteriophages such as λ -phage or their derivatives, animal viruses such as retrovirus, vaccinia virus etc., and insect viruses.

The 5'-terminal of said gene may possess ATG as the initiation codon, and the 3'-terminal may possess TAA, TGA or TAG as the termination codon. To express said gene, a promoter is linked to an upstream region. The promoter used in the present invention may be any promoter if it is suitable for a host used in gene expression. Examples of promoters are follows: a trp promoter, lac promoter, rec A promoter, λ PL promoter, lpp promoter, T7 promoter etc. for *E. coli* as the host transformed; SP01 promoter, SP02 promoter, penP promoter, etc. for *Bacillus subtilis* as the host; and PH05 promoter, PGK promoter, GAP promoter, ADH promoter etc. for yeast as the host. If the host is animal cells, SV40-derived promoter, retrovirus promoter etc. are used.

The thus constructed vector containing the nucleotide sequence coding for the FGF-5 analogous protein is used to produce a transformant carrying said vector.

The host includes *E. coli* (e.g. BL21, BL21 (DE3), BL21 (DE3) pLsS, BL21(DE3) pLysE), *Bacillus subtilis* (e.g.*Bacillus subtilis* DB105), yeast (e.g.*Pichia pastoris, Saccharomyces cerevisiae*), animal cells (e.g. COS cell, CHO cell, BHK cell, NIH3T3 cell, BALB/c3T3 cell, HUVE cell, LEII cell), and insect cells.

The transformation may be carried out in a usual manner. A peculiar method can also be used if it is applicable. If the host is *E. coli*, heat shock or electroporation is used to introduce the vector containing the recombinant DNA into competent cells prepared in the calcium method or any other method. If the host is yeast, heat shock or electroporation is used to introduce the vector containing the recombinant DNA into competent cells prepared in the lithium method or any other method. If the host is animal cells, the calcium phosphate method, lipofection method or electroporation is used to introduce the vector containing the recombinant DNA into cells at the proliferation phase etc.

The FGF-5 analogous protein is produced by culturing the resulting transformant in a medium.

To culture the transformant, the medium is that generally used for a desired host. A peculiar method can also be used if it is applicable. If the host is *E. coli*, LB medium etc. may be used. If the host is yeast, YPD medium etc. may be used. If the host is animal cells, Dulbecco's MEM supplemented with animal serum etc. may be used. The culture is carried under conventional conditions for a desired host. Peculiar conditions may also be used if they are applicable. If the host is *E. coli*, the culture is carried out at about 30° to 37° C. for about 3 to 24 hours, if necessary under aeration or stirring. If the host is yeast, the culture is carried out at about 25° to 37° C. for about 12 hours to 2 weeks, if necessary under aeration or stirring. If the host is animal cells, the culture is carried out at about 32° to 37° C. in 5% CO_2 and 100% humidity for about 24 hours to 2 weeks, if necessary under stirring, and the conditions for the gaseous phase may be changed if required.

After the culture, the microorganism or cells are disrupted with a homogenizer, a French press, ultrasonication, lysozyme and/or freezing and thawing to elute FGF-5 analogous protein outside, and this protein can be obtained from the soluble fraction. If the target protein is contained in the insolubilized fraction, the microorganism or cells are disrupted and the insolubilized fraction is recovered by centrifugation, and the protein can be solubilized with e.g. a buffer containing guanidine hydrochloride. Alternatively, the microorganism or cells are disrupted directly with a buffer containing a protein denaturant such as guanidine hydrochloride etc. to elute the target protein outside.

To purify the FGF-5 analogous protein from the supernatant obtained above, a suitable combination of conventional separation and purification means can be used. Such conventional separation and purification means include salting-out, solvent precipitation, dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, ion-exchange chromatography, affinity chromatography, reverse phase high performance liquid chromatography, and isoelectric focusing. Affinity chromatography on heparin Sepharose as the carrier can further be applied to a large number of FGF-5 analogous proteins.

The preparation thus obtained may be dialyzed and lyophilized into dry powder unless the activity of the FGF-5 analogous protein is lost. The addition of serum albumin as a carrier can effectively prevent the adsorption of the preparation onto the vessel in storage.

In addition, the addition of a trace amount of a reducing agent in the purification process or in storage is effective for preventing oxidation of the preparation. The reducing agent used is β -mercaptoethanol, dithiothreitol, glutathione etc.

The FGF-5 analogous protein of the present invention regulates the mechanism of hair production, specifically for promoting or suppressing restoration of hairs on the head or promoting or suppressing hair growth.

Further, the FGF-5 analogous protein of the present invention regulates nutrition and functions in the brain nerve system.

Furthermore, the FGF-5 analogous protein of the present invention regulates the physiological functions of FGF-5 in addition to said actions. The physiological functions of FGF-5 refer specifically to promotion or suppression of proliferation or differentiation of fibroblasts, vascular endothelial cells, myoblasts, chondrocytes, osteoblasts and glia cells, or to regulation of the functions of these cells.

By virtue of said functions of the FGF-5 analogous protein, this protein is useful for prevention and treatment of various diseases, such as fibroblastoma, hemangioma, osteoblastoma, nerve cell death, Alzheimer's disease, Parkinson's disease, neuroblastoma, amnesia, dementia and myocardial infarction.

The FGF-5 analogue protein obtained as described above makes use of a pharmaceutical acceptable solvent, fillers, carriers and auxiliary agents to be formed in a usual manner into a pharmaceutical composition in the form of a liquid, lotion, aerosol, injection, powder, granules, tablets, suppositories, intestinal agent, capsules etc.

In the pharmaceutical composition, the content of the FGF-5 analogous protein as the active ingredient may range from 10^{-10} to 1.0% by weight.

The pharmaceutical composition can be administrated orally or parenterally and safely into mammals such as humans, mouses, rats, rabbits, dogs, cats etc. as a hair restoration agent, hair development agent, nutrition and functional suppresser in the brain and nervous system, learning effect regulator etc. The dose of the present pharmaceutical composition can be varied depending on the form of the agent, administration route, symptoms etc. For example, 10^{-4} to 10^{3} mg of the FGF-5 analogue protein may be administrated several times per day.

Deposition of the Microorganism

E. coli MFGF5S/PBS/XL1 carrying a plasmid containing the gene of the DNA sequence of FIG. 2 coding for mouse FGF-5 analogous protein according to the present invention has been deposited since Mar. 12, 1996 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

EXAMPLES

Hereinafter, the present invention is described in detail by reference to the following examples, which however are not intended to limit the present invention.

Example 1

Preparation of cDNA coding for human FGF-5 analogue (human FGF-5S)

Human RNA, e.g. human brain whole RNA (Catalog No. 640202-1, available from Clontech) is used. To 1 μg of this human RNA was added 120 ng random hexanucleotide DNA, and reverse transcription was carried out using 200 units of M-HLV reverse transcriptase in 50 mM Tris-HCl buffer, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.4 mM dNTPs and 40 units of RN asin to give a cDNA mixture.

Then, the target gene in the cDNA mixture thus obtained was amplified by PCR reaction in the following manner. The cDNA mixture, 0.5 μ1, was subjected to amplification with 0.5 unit of AmpliTaq (Perkin Elmer) in the presence of 250 μM dNTPs, 75 mM NH_4 O_2 SO_4, pH 8.5, 2.0 mM $MgCl_2$, 0.5 μg sense primer (A, shown below) and 0.5 μg antisense primer (B, below) in 40 cycles each consisting of thermal treatment at 94° C. for 1 minute, 60° C. for 2 minutes and 72° C. for 1 minute.

A: 5'-GA ATG AGC TTG TCC TTC CTC CTC CTC CTC TTC TTC AGC CAC-3' (SEQ ID NO:5)

B: 5'-AAG TTC TGG CTG CTC CGA CTG CTT-3' (SEQ ID NO:6)

As a result, cDNA coding for human FGF-5S was formed. The formed cDNA was cloned in pBlueScript vector and its nucleotide sequence was confirmed (FIG. 1).

Example 2

Preparation of cDNA Coding for Mouse FGF-5 Analog (Mouse FGF-5S)

The brain was isolated from a female ICR mouse, 6-week-old, and RNA was extracted from it. 120 ng random hexanucleotide DNA was added to 1 μg of the mouse RNA, and reverse transcription was carried out using 200 units of M-HLV reverse transcriptase in 50 mM Tris-HCl buffer, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.4 mM dNTPs and 40 units of RN asin to give a cDNA mixture.

Then, the target gene in the cDNA mixture thus obtained was amplified by PCR reaction in the following manner. The cDNA mixture, 0.5 μ1, was subjected to amplification with 0.5 unit of AmpliTaq (Perkin Elmer) in the presence of 250 μM dNTPs, 75 mM NH_4 O_2 SO_4, pH 8.5, 2.0 mM $MgCl_2$, 0.5 μg sense primer (C, shown below) and 0.5 μg antisense primer (D, below) in 40 cycles each consisting of thermal treatment at 94° C. for 1 minute, 60° C. for 2 minutes and 72° C. for 1 minute.

C: 5'-AAGAATGAGCCTGTCCTTGCTCTTCCTCATCTTCTGCAGCCACCTGATCCA-3' (SEQ ID NO:7)

D: 5'-AAGTTCCGGTTGCTCGGACTGCTT-3' (SEQ ID NO:8)

As a result, cDNA coding for the target mouse FGF-5S was formed. The formed cDNA was cloned in pBlueScript vector, and its nucleotide sequence was confirmed (FIG.2).

Example 3

Expression a nd Identi fication of Mouse FGF-5 Analog

As a template, the vector carrying the cDNA coding for mouse FGF-5S i nserted into a region downstream of T7 promoter was transcribed and translated in a usual manner, and the translational product was separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. This membrane was incubated with an anti-FGF-5 rabbit antibody and the antibody-bound molecule was detected with a HRP-labeled anti-rabbit antibody in a chemiluminescence method. The results are shown in FIG. 3. Lane 1 is the prepared FGF-5S protein, and lane 2 is whole-length FGF-5 produced by *E. coli*. It was con-

firmed that the FGF-5S cDNA codes for the protein molecule with a molecular weight of about 14,000 and the protein has an epitope in common with FGF-5, and simultaneously the protein was obtained.

Example 4

Confirmation of Expression of FGF-5 Analog (FGF-5S) mRNA by RNase Protection Assay

Mouse FGF-5 cDNA inserted into pBluescript vector (Stratagene) was transcribed in the presence of labeled RNA precursors to synthesize a 652-bp labeled riboprobe capable of hybridizing to a part of FGF-5 mRNA corresponding to the whole region of exons 1, 2 and 3 in FGF-5 gene. Whole RNA was extracted from mouse cells derived from the nerve cells, then mixed with and hybridized to the above labeled riboprobe under suitable conditions, and the RNA not hybridized to the probe was removed by digestion with RNase T1. The hybridized RNA was electrophoresed to determine molecular weight. As a result, 326-bp and 222-bp reaction products showing the presence of the FGF-5 analog (FGF-5S) mRNA were detected along with a 652-bp reaction product showing the presence of the FGF-5 mRNA. This indicated the presence of the FGF-5 analog (FGF-5S) mRNA in nerve cells.

Example 5

Expression of FGF-5 Analog (FGF-5S) Protein in *E. coli* and Its Purification

Human FGF-5 analog (FGF-5S) cDNA was inserted into a region downstream from a sequence coding for maltose-binding protein (MBP) contained in fusion protein expression plasmid vector pMAL-c2 (New England Biolabs) to construct expression plasmid pMAL/humFGF5S. *E. coli* was transformed with this plasmid and cultured in a liquid medium. The expression of a MBP-FGF5S fusion protein was induced by adding isopropylgalactopyranoside in the latter period of culture. The microorganism was disrupted by ultrasonication to release the fusion protein, and this fusion protein was purified through an affinity column on amylose resin (New England Biolabs). The fusion protein thus obtained was cleaved with a specific proteolytic enzyme Factor Xa (New England Biolabs) to give MBP and FGF-5 analog (FGF-5S) protein, and the FGF-5 analog protein was then separated. As a result, high-purity FGF-5 analog (FGF-5S) protein could be obtained in a large amount.

Example 6

Regulatory Action of FGF-5 Analog (FGF-5S) Protein on Cell Proliferation Induced by FGF-5 Protein

The FGF-5 protein was added to a culture liquid of cultured fibroblast mouse 3T3 cells under serum starvation, and cell proliferation was evaluated by determining the amount of DNA synthesized by the cells from 15 to 19 hours after the initiation of culture. As a result, the amount of synthesized DNA was increased depending on the amount of the FGF-5 protein added to the culture, indicating that the FGF-5 protein possessed cell proliferation activity. On the other hand, the synthesis of DNA by cells was inhibited when the cells were cultured in the presence of both the FGF-5 protein and FGF-5 analog (FGF-5S) protein. This is an example where the FGF-5 analog (FGF-5S) protein regulates the proliferation of cells by the FGF-5 protein.

Example 7

Regulatory Action of FGF-5 Analog (FGF-5S) Protein on Neurotrophic Activity of FGF-5

Human FGF-5 analog (FGF-5S) cDNA and human FGF-5 cDNA were inserted into cloning sites in animal cell expression vector pMEXneo to construct expression plasmids pMEX/FGF-5S and pMEX/FGF-5, respectively. PC12 cells, i.e. model cells for nerve differentiation, were transformed with either or both of plasmids pMEX/FGF-5S and pMEX/FGF-5 and examined for their acetylcholine esterase activity. This acetylcholine esterase activity serves as an indicator for the nerve differentiation of PC12 cells and can thus be used to evaluate neurotrophic activity. In this experiment, the PC12 cells having the FGF-5 solely showed nerve differentiation, while the PC12 cells having both the FGF-5 and FGF-5 analog (FGF-5S) hardly showed nerve differentiation. This is an example where the FGF-5 analog (FGF-5S) regulates trophic activity of FGF-5 on nervous system.

Effect of the Invention

According to the pharmaceutical composition with the novel FGF-5 analogous protein of the present invention as an active ingredient, it is possible to regulate the physiological functions of FGF-5, such as regulation of restoration or development of hairs on the head or body, trophic or functional regulation in the brain nerve system, proliferation of fibroblasts, etc.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 8

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 372 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) FEATURE:
(A) NAME/KEY: None
(B) LOCATION: 1...369

-continued

(D) OTHER INFORMATION:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATGAGCTTGT CCTTCCTCCT CCTCCTCTTC TTCAGCCACC TGATCCTCAG CGCCTGGGCT    60
CACGGGGAGA AGCGTCTCGC CCCCAAAGGG CAACCCGGAC CCGCTGCCAC TGATAGGAAC   120
CCTATAGGCT CCAGCAGCAG ACAGAGCAGC AGTAGCGCTA TGTCTTCCTC TTCTGCCTCC   180
TCCTCCCCCG CAGCTTCTCT GGGCAGCCAA GGAAGTGGCT TGGAGCAGAG CAGTTTCCAG   240
TGGAGCCCCT CGGGGCGCCG GACCGGCAGC CTCTACTGCA GAGTGGGCAT CGGTTTCCAT   300
CTGCAGATCT ACCCGGATGG CAAAGTCAAT GGATCCCACG AAGCCAATAT GTTAAGCCAA   360
GTTCACAGAT GA                                                       372
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Leu Ser Phe Leu Leu Leu Leu Phe Phe Ser His Leu Ile Leu
 1               5                  10                  15

Ser Ala Trp Ala His Gly Glu Lys Arg Leu Ala Pro Lys Gly Gln Pro
            20                  25                  30

Gly Pro Ala Ala Thr Asp Arg Asn Pro Ile Gly Ser Ser Ser Arg Gln
        35                  40                  45

Ser Ser Ser Ser Ala Met Ser Ser Ser Ser Ala Ser Ser Ser Pro Ala
    50                  55                  60

Ala Ser Leu Gly Ser Gln Gly Ser Gly Leu Glu Gln Ser Ser Phe Gln
65                  70                  75                  80

Trp Ser Pro Ser Gly Arg Arg Thr Gly Ser Leu Tyr Cys Arg Val Gly
                85                  90                  95

Ile Gly Phe His Leu Gln Ile Tyr Pro Asp Gly Lys Val Asn Gly Ser
            100                 105                 110

His Glu Ala Asn Met Leu Ser Gln Val His Arg
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 366 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) FEATURE:
(A) NAME/KEY: None
(B) LOCATION: 1..363
(D) OTHER INFORMATION:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATGAGCCTGT CCTTGCTCTT CCTCATCTTC TGCAGCCACC TGATCCACAG CGCTTGGGCT    60
CACGGGGAGA AGCGTCTCAC TCCCGAAGGG CAACCCGCGC CTCCTAGGAA CCCGGGAGAC   120
TCCAGCGGCA GCCGGGGCAG AAGTAGCGCG ACGTTTTCTT CGTCTTCTGC CTCCTCACCA   180
GTCGCAGCTT CTCCGGGCAG CCAAGGAAGC GGCTCGGAAC ATAGCAGTTT CCAGTGGAGC   240
```

-continued

```
CCTTCGGGGC GCCGGACCGG CAGCCTGTAC TGCAGAGTGG GCATCGGTTT CCATCTGCAG   300

ATCTACCCGG ATGGCAAAGT CAATGGCTCC CACGAAGCCA GTGTGTTAAG CCAAATTTAC   360

GGATGA                                                              366
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ser Leu Ser Leu Leu Phe Leu Ile Phe Cys Ser His Leu Ile His
 1               5                  10                  15

Ser Ala Trp Ala His Gly Glu Lys Arg Leu Thr Pro Glu Gly Gln Pro
            20                  25                  30

Ala Pro Pro Arg Asn Pro Gly Asp Ser Ser Gly Ser Arg Gly Arg Ser
        35                  40                  45

Ser Ala Thr Phe Ser Ser Ser Ser Ala Ser Ser Pro Val Ala Ala Ser
    50                  55                  60

Pro Gly Ser Gln Gly Ser Gly Ser Glu His Ser Ser Phe Glu Trp Ser
65                  70                  75                  80

Pro Ser Gly Arg Arg Thr Gly Ser Leu Tyr Cys Arg Val Gly Ile Gly
                85                  90                  95

Phe His Leu Gln Ile Tyr Pro Asp Gly Lys Val Asn Gly Ser His Glu
            100                 105                 110

Ala Ser Val Leu Ser Gln Ile Tyr Gly
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GAATGAGCTT GTCCTTCCTC CTCCTCCTCT TCTTCAGCCA C                       41
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
AAGTTCTGGC TGCTCCGACT GCTT                                          24
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGAATGAGC CTGTCCTTGC TCTTCCTCAT CTTCTGCAGC CACCTGATCC A 51

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGTTCCGGT TGCTCGGACT GCTT 24

What is claimed is:

1. An isolated and purified FGF-5 protein having the amino acid sequence as set forth in SEQ ID NO: 2.

2. An isolated and purified FGF-5 protein having the amino acid sequence as set forth in SEQ ID NO: 4.

3. An isolated and purified DNA molecule having a nucleotide sequence coding for the amino acid sequence as set forth in SEQ ID NO: 2.

4. An isolated and purified DNA molecule having a nucleotide sequence coding for the amino acid sequence as set forth in SEQ ID NO: 4.

5. An isolated and purified DNA molecule having the nucleotide sequence as set forth in SEQ ID NO: 1.

6. An isolated and purified DNA molecule having the nucleotide sequence as set forth in SEQ ID NO: 3.

7. A pharmaceutical composition comprising the protein of claim 1 and a pharmaceutically acceptable carrier.

8. A pharmaceutical composition comprising the protein of claim 2 and a pharmaceutically acceptable carrier.

* * * * *

US006638502B1

(12) **United States Patent**
Li et al.

(10) **Patent No.: US 6,638,502 B1**
(45) **Date of Patent: Oct. 28, 2003**

(54) **ADENOVIRUS-MEDIATED INTRATUMORAL DELIVERY OF AN ANGIOGENESIS ANTAGONIST FOR THE TREATMENT OF TUMORS**

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(73) Assignee: **Gencell SAS**, Vitry sur Seine (FR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/403,736**

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§ 371 (c)(1),
(2), (4) Date: **Jun. 29, 2000**

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(52) **U.S. Cl.** **424/93.2**; 514/44; 435/455; 435/456; 435/320.1

(58) **Field of Search** 514/44; 536/23.5; 435/235.1, 456, 320.1, 455; 424/93.2

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,650,764	A	3/1987	Temin et al.	435/350
4,797,368	A	1/1989	Carter et al.	435/320.1
4,814,470	A	3/1989	Colin et al.	514/449
4,857,653	A	8/1989	Colin et al.	549/511
4,861,719	A	8/1989	Miller	435/236
4,924,011	A	5/1990	Denis et al.	549/510
4,980,289	A	12/1990	Temin et al.	435/235.1
5,124,263	A	6/1992	Temin et al.	435/349
5,139,941	A	8/1992	Muzyczka et al.	435/456
5,168,062	A	12/1992	Stinski	435/366
5,290,957	A	3/1994	Correa et al.	549/510
5,292,921	A	3/1994	Correa et al.	560/29
5,385,839	A	1/1995	Stinski	435/366
5,399,346	A	3/1995	Anderson et al.	424/93.21
5,438,072	A	8/1995	Bobee et al.	514/449
5,459,127	A	10/1995	Felgner et al.	514/7
5,580,859	A	12/1996	Felgner et al.	514/44
5,587,493	A	12/1996	Bouchard et al.	549/510
5,589,466	A	12/1996	Felgner et al.	514/44
5,639,725	A	6/1997	O'Reilly et al.	514/12
5,652,095	A *	7/1997	Taniguchi et al.	435/6
5,885,795	A *	3/1999	O'Reilly et al.	435/69.1
6,080,728	A *	6/2000	Mixson	514/44
6,143,290	A *	11/2000	Zhang et al.	424/93.2
6,251,433	B1 *	6/2001	Zuckermann et al.	424/486

FOREIGN PATENT DOCUMENTS

CA	2012311	3/1990
EP	185 573	6/1986
EP	253 738	7/1987
EP	453 242	4/1990
EP	178 220	2/1992
EP	488 528	11/1995
FR	2726285	5/1996
WO	WO 89/07150	8/1989
WO	WO 90/02806	3/1990
WO	WO 91/17976	11/1991
WO	WO 91/18088	11/1991
WO	WO 92/05263	4/1992
WO	WO 93/00928	1/1993
WO	WO 93/00929	1/1993
WO	WO 93/09239	5/1993
WO	WO 93/15199	8/1993
WO	WO 94/12649	6/1994
WO	WO 95/18863	7/1994
WO	WO 94/21807	9/1994
WO	WO 94/26914	11/1994
WO	WO 94/28152	12/1994
WO	WO 94/28938	12/1994
WO	WO 95/02697	1/1995
WO	WO 95/07358	3/1995
WO	WO 95/21931	8/1995
WO	WO 95/28494	10/1995
WO	WO 95/29242	11/1995
WO	WO 96/01313	1/1996
WO	WO 96/01815	1/1996
WO	WO 96/17823	6/1996
WO	WO 96/22378	7/1996
WO	WO 96/25508	8/1996
WO	WO 97/04092	2/1997
WO	WO 97/15666	5/1997

OTHER PUBLICATIONS

Wilson, Vehicles for gene therapy, 1993, NAUTRE, vol. 365, pp. 691–692.*
Tanaka et al., "Viral vector–mediated transduction of modified platelet factor 4 cDNA inhibits angiogenesis and tumor growth," Nature Med. 3 (4): 437–442, Apr. 5, 1997.*

(List continued on next page.)

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(57) **ABSTRACT**

The present invention relates to gene therapy for the treatment of tumors. The invention more particularly relates to introduction of a gene encoding an anti-angiogenic factor into cells of a tumor, for example with a defective adenovirus vector, to inhibit growth or metastasis, or both, of the tumor. In a specific embodiment, delivery of a defective adenovirus that expresses the amino terminal fragment of urokinase (ATF) inhibited growth and metastasis of tumors. These effects were correlated with a remarkable inhibition of neovascularization within, and at the immediate vicinity of, the injection site. Delivery of a defective adenovirus vector that expresses kringles 1 to 3 of angiostatin inhibited tumor growth and tumorigenicity, and induced apoptosis of tumor cells. The invention further provides viral vectors for use in the methods of the invention.

14 Claims, 11 Drawing Sheets

AD-ATF (103)

OTHER PUBLICATIONS

Tanaka et al., "Retrovral and adenoviral mediated transduction fo angiostatin cDNA inhibits angiogenesis and tumor growth," Proc. Amer. Assoc. Cancer Res. 38: 264, Mar. 1997.*

Dang et al.Gene Therapy and Translational Cancer Therapy, Clinical Cancer Research, vol. 5, 471–474, Feb. 1999.*

Eck, S.L. et al., 1996, Ch. 5. Gene Based Therapy,Goodman & Gillman's The Pharmacological basis of Therapeutics. p. 77–101ck, et al.*

Robbins, Review: Viral Vectors for gene therapy, Trends in Biotechnology, vol. 16, pp. 35–40.*

Estreicher et al., The Receptor for Urokinase Type Plasminogen Activator Polarizes Expression of the Protease to the Leading Edge of Migrating Monocytes and Promotes Degradation of Enzyme Inhibitor Complexes, Journal of Cell Biology 111, 783–792 (1990).

Pepper et al., Chondrocytes Inhibit Endothelial Sprout Formation In Vitro: Evidence for Involvement of a Transforming Growth Factor–Beta, Journal of Cellular Physiology 146, 170–179 (1991).

Ploug et al., Cellular Receptor for Urokinase Plasminogen Activator, The Journal of Biological Chemistry 266(3), 1926–1933 (1991).

Ellis et al., Plasminogen Activation by Receptor–bound Urokinase, The Journal of Biological Chemistry 266(19), 12752–12758 (1991).

Wei et al., Identification of the Urokinase Receptor as an Adhesion Receptor for Vitronectin, The Journal of Biological Chemistry 269(51), 32380–32388 (1994).

Appella et al., The Receptor–binding Sequence of Urokinase: A Biological Function for the Growth–Factor Module of Proteases, The Journal of Biological Chemistry 262(10), 4437–4440 (1987).

Cao et al., Kringle Domains of Human Angiostatin, The Journal of Biological Chemistry 271(46), 29461–29467 (1996).

Imren et al., Overexpression of Tissue Inhibitor of Metalloproteinases–2 by Retroviral–mediated Gene Transfer in Vivo Inhibits Tumor Growth and Invasion, Cancer Research 56, 2891–2895 (1996).

Shapiro et al., Induction of Primary Cutaneous Melanocytic Neoplasms in Urokinase–Type Plasminogen Activator (uPA)–deficient and Wild–Type Mice: Cellular Blue Nevi Invade but Do Not Progress to Malignant Melanoma in uPA–deficient Animals, Cancer Research 56, 3597–3604 (1996).

Skladanowski et al., Expression of Wild–Type p53 Increases Etopside Cytotoxicity in M1 Myeloid Leukemia Cells by Facilitated G2 to M Transtition: Implication for Gene Therapy, Cancer Research 57, 818–823 (1997).

Sim et al., A Recombinant Human Angiostatin Protein Inhibits Experimental Primary and Metastatic Cancer, Cancer Research 57, 1329–1334 (1997).

Liotta et al., Quantitative Relationships of Intravascular Tumor Cells, Tumor Vessels, and Pulmonary Metastases folling Tumor Implantation, Cancer Research 34, 997–1004 (1974).

Hori et al., Suppression of Solid Tumor Growth by Immunoneutralizing Monoclonal Antibodies against Human Basic Fibroblast Growth Factor, Cancer Research 51, 6180–6184 (1991).

Min et al., Urokinase Receptor Antagonists Inhibit Angiogenesis and Primary Tumor in Syngeneic Mice, Cancer Research 56, 2428–2433 (1996).

Wei et al., Regulation of Integrin Function by the Urokinase Receptor, Science 273, 1551–1555 (1996).

Graeber et al., Hypoxia–mediated selection of cells with diminished apoptotic potential in solid tumours, Nature 379, 88–91 (1996).

Bacharach et al., In vivo patterns of expression of urokinase and its inhibitor PAI–1 suggest a concerted role in regulating physiological angiogenesis, Proc. Natl. Acad. Sci. USA 89: 10686–10690 (1989).

He et al., Tissue cooperation in a proteolytic cascade activating human interstitial collagenase, Proc. Natl. Acad. Sci. 86, 2632–2636 (1989).

Crowley et al., Prevention of metastasis by inhibition of the urokinase receptor, Proc. Natl. Acad. Sci. 90, 5021–5025 (1993).

Davis et al., Monoclonal antibodies to mitotic cells, Proc. Natl. Acad. Sci. 80, 2926–2930 (1983).

Gupta et al., A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4, Proc. Natl. Acad. Sci 92, 7799–7803 (1995).

Mignatti et al., Biology & Biochemistry of Proteinases in Tumor Invasion, Physiological Reviews 73(1), 161–195 (1993).

Pyke et al., Urokinase–type Plasminogen Activator is Expressed in Stromal Cells and Its Receptor in Cancer Cells and Its Receptor in Cancer Cells at Invasive Foci in Human Colon Adenocarcinomas, American Journal of Pathology 138(5), 1059–1067 (1991).

Magdolen et al., Systematic mutational analysis of the receptor–binding region of the human urokinase–type plasminogen activator, Eur J. Biochem 237, 743–751 (1996).

Schmitt et al., Tumor–associated Proteases, Fibrinolysis 6(Suppl. 1), 3–26 (1992).

Stratford–Perricaude et al., Widespread Long–term Gene Transfer to Mouse Skeletal Muscles and Heart, J. Clin. Invest. 90, 626–630 (1992).

Weidner et al., Tumor Angiogenesis and Metastasis–Correlation in Invasive Breast Carcinoma, The New England Journal of Medicine 324(1), 1–8 (1991).

Hanahan et al., Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis, Cell 86, 353–364 (1996).

Wickham et al., Integrins avB3 and avB5 Promote Adenovirus Internalization but Not Virus Attachment, Cell 73, 309–319 (1993).

Brooks et al., Integrin avB3 Antagonists Promote Tumor Regression by Inducing Apoptosis of Angiogenic Blood Vessels, Cell 79, 1157–1164 (1994).

O'Reilly et al., Endostatin: An Endogenous Inhibitor of Angiogenesis and Tumor Growth, Cell 88, 277–285 (1997).

Dong et al., Macrophage–Derived Metalloelastase Is Responsible for the Generation of Angiostatin in Lewis Lung Carcinoma, Cell 88, 801–810 (1997).

King et al., Mitosis in Transition, Cell 79, 563–571 (1994).

Yeh et al., Advances in adenoviral vectors: from genetic engineering to their biology, FASEB 11, 615–623 (1997).

Folkman, What Is the Evidence That Tumors are Angiogenesis Dependent?, Journal of the National Cancer Institute 82(1), 4–6 (1990).

Mirshahi et al., Evaluation of the Inhibition by Heparin and Hirudin of Coagulation Activation During r-tPA-Induced Thrombolysis, Blood, 74(3), 1025–1030 (1989).

Plate et al., Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo, Nature 359, 845–848 (1992).

Roth et al., Gene Therapy for Cancer: What Have We Done and Where Are We Going?, Journal of the National Cancer Institute 89(1), 21–39 (1997).

Clapp et al., The 16-Kilodalton N-Terminal Fragment of Human Prolactin Is a Potent Inhibitor of Angiogenesis, Endocrinology 133(3), 1292–1299 (1993).

Kim et al., Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo, Nature 362, 841–844 (1993).

Hamada et al., Separable groth and migration factors for large-cell lymphoma cells secreted by microvascular endothelial cells derived from target organs for metastasis, Br. J. Cancer 66, 349–354 (1992).

Trochon et al., Evidence of Involvement of CD44 In Endothelial Cell Proliferation, Migration and Angiogenesis In Vivo, Int. J. Cancer 66, 664–668 (1996).

Fan et al., Controlling the vasculature: angiogensis, anti-angiogenesis and vascular targeting of gene therapy, TiPS 16, 57–66 (1995).

Rifkin et al., Growth factor control of extracellular proteolysis, Cell Differentiation & Development 32, 313–318 (1990).

Lu et al., Blockage of urokinase receptor reduces in vitro the motility and the deformability of endothelial cells, FEBS Letters 380, 21–24 (1996).

Kobayashi et al., Inhibition of Metastasis of Lewis Lung Carcinoma by A Synthetic Peptide Within Grown Factor-Like Domain of Urokinase In The Experimental and Spontaneous Metastasis Model, Int. J. Cancer 57, 727–733 (1994).

Lu et al., Blockage of the urokinase receptor on the cell surface: construction and characterization of a hybrid protein consisting of the N-terminal fragment of human urokinase and human albumin, FEBS Letters 356, 56–59 (1994).

Holmegren et al., Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression, Nature Medicine 1(2), 149–153 (1995).

Graham et al., Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5, J. Gen. Virol. 36, 59–72 (1977).

Hayes et al., Carbohydrate of the Human Plasminogen Variants, The Journal of Biological Chemistry 254(18), 8772–8776 (1979).

Roldan et al., Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis, The EMBO Journal 9(2), 467–474 (1990).

Hirt, Selective Extraction of Polyoma DNA from Infected Mouse Cell Cultures, J. Mol. Biol. 26, 365–369 (1967).

Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease, Nature Medicine 1(1), 27–31 (1995).

Belin et al., Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator, Eur. J. Biochem 148, 225–232 (1985).

Naldini et al., Extracellular proteolytic cleavage by urolinase is required for activation of hepatocyte growth factor/scatter factor, The EMBO Journal 11(13), 4825–4833 (1992).

O'Reilly et al., Angiostatin induces and sustains dormancy of human primary tumors in mice, Nature Medicine 2(6), 689–692 (1996).

Markowitz et al., A Safe Packaging Line for Gene Transfer: Separating Viral Genes on Two Different Plasmids, Journal of Virology 62(4), 1120–1124 (1988).

Kuo et al., Efficient Gene Transfer Into Primary Murine Lymphocytes Obviating the Need for Drug Selection, Blood 82(3), 845–852 (1993).

Machy et al., Gene transfer from targeted liposomes to specific lymphoid cells by electroporation, Proc. Natl. Acad. Sci. 85, 8027–8031 (1988).

Felgner et al., Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl. Acad. Sci. 84, 7413–7417 (1987).

Bender et al., Evidence that the Packaging Signal of Moloney Murine Leukemia Virus Extends into the gag Region Journal of Virology 1639–1646 (1987).

Ulmer et al., Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein, Science 259, 1745–1749 (1993).

Le Gal La Salle et al., An Adenovirus Vector for Gene Transfer into Neurons and Glia in the Brain, Science 259, 988–990 (1993).

Samulski et al., A Recombinant Plasmid from Which an Infectious Adeno-Associated Virus Genome can be Excised in Vitro and Its use To Study Viral Replication, Journal of Virology 61(10), 3096–3101 (1987).

Dvorak et al., Iidentification and Characterization of the Blood Vessels of Solid Tumors That Are Leaky to Circulating Macromolecules, American Journal of Pathology 133(1), 95–109 (1988).

Readhead et al., Expression of a Myelin Basic Protein Gene in Transgenic Shiverer Mice: Correction of the Dysmyelinating Phenotype, Cell, 48, 703–712 (1987).

Kollias et al., Regulated Expression of Human A Gamma, Beta, and Hybrid Gamma Beta-Globin Genes in Trasgenic Mice: Manipulation of the Developmental Expression Patterns, Cell 46, 89–94, (1986).

Leder et al., Consequences of Widespread Deregulation of the c-myc Gene in Transgenic Mice: Multiple Neoplasms and Normal Development, Cell 45, 485–495 (1986).

Yamamoto et al., Identification of a Functional Promoter in the Long Terminal Repeat of Rous Sarcoma Virus, Cell 22, 787–797 (1980).

Mann et al., Construction of a Retrovirus Packaging Mutant and Its use to Produce Helper-Free Defective Retrovirus, Cell 33, 153–159 (1983).

Swift et al., Tissue-Specific Expression of the Rat Pancreatic Elastase I Gene in Transgenic Mice. Cell 38, 639–646 (1984).

Grosschedl et al., Introduction of a Micro Immunoglobulin Gene into the Mouse Germ Line: Specific Expression in Lymphoid Cells and Synthesis of Functional Antibody, Cell 38, 647–658 (1984).

Lebkowski et al., Adeno-Associated Virus: a Vector system for Efficient Introduction and Integration of DNA into a Variety of Mammalian Cell Types, Molecular & Cellular Biology 8(10), 3988–3996 (1988).

Alexander et al., Expression of the c-myc Oncogene under Control of an Immunoglobulin Enhancer in Eu-myc Transgenic Mice, Molecular & Cellular Biology 7(4), 1436–1444 (1987).

Krumlauf et al., Developmental Regulation of alpha–Fetoprotein Genes in Transgenic Mice, Molecular & Cellular Biology 5(7), 1639–1648 (1985).

Hammer et al., Diversity of Alpha–Fetoprotein Gene Expression in Mice Is Generated by a Combination of Separate Enhancer Elements, Science 235, 53–58 (1987).

Mason et al., The Hypogonadal Mouse: Reproductive Functions Restored by Gene Therapy, Science 234, 1372–1378 (1986).

Williams et al., Introduction of foreign genes into tissues of living mice by DNA–coated microprojectiles, Pro. Natl. Acad. Sci. 88, 2726–2730 (1991).

DeBoer et al., The tac promoter: A functional hybrid derived from the trp and lac promoters, Proc. Natl. Acad. Sci. 80, 21–25 (1983).

Villa–Komaroff et al., A bacterial clone synthesizing proinsulin, Proc. Natl. Acad. Sci. 75(8), 3727–3731 (1978).

Wagner et al., Nucleotide sequence of the thymidine kinase gene or herpes simplex virus type 1, Proc. Natl. Acad. Sci. 78(3), 1441–1445 (1981).

Hutchison et al., A complete library of point substitution mutations in the glucocorticoid response element of mouse mammary tumor virus, Proc. Natl. Acad. Sci. 83, 710–714 (1986).

Wu et al., Receptor–mediated Gene Delivery and Expression in Vivo, Journal of Biological Chemistry 263(29), 14621–14624 (1988).

Wilson et al., Hepatocyte–directed Gene Transfer in Vivo Leads to Transient Improvement of Hypercholesterolemia in Low Density Lipoprotein Receptor–deficient Rabbits, Journal of Biological Chemistry 267(2), 963–967 (1992).

Wu et al., Receptor–mediated in Vitro Gene Transformation by a Soluble Carrier DNA System, Journal of Biological Chemistry 262(10), 4429–4432 (1987).

Benoist et al., In vivo sequence requirements of th SV40 early promoter region, Nature 290, 304–310 (1981).

Brinster et al., Regulation of metallothinonein–thymidine kinase fusion plasmids injected into mouse eggs, Nature 296, 39–42 (1982).

Shani, Tissue–specific expression of rat myosin light–chain 2 gene in transgenic mice, Nature 314, 283–286 (1985).

Hanahan, Heritable formation of pancreatice Beta–cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes, Nature 315, 115–122 (1985).

Magram et al., Developmental regulation of a cloned adult Beta–globin gene in transgenic mice, Nature 315, 338–340 (1985).

Gilbert et al., Useful Proteins from Recombinant Bacteria, Scientific American 242(4), 74–94 (1980).

Kaplitt et al., Expression of a Functional Foreign Gene in Adult Mammalian Brain following in Vivo Transfer via a Herpes Simplex Virus Type 1 Defective Viral Vector, Molecular & Cellular Neurosciences 2, 320–330 (1991).

Samulski et al., Helper–Free Stocks of Recombinant Adeno–Associated Viruses: Normal Integration Does Not Require Viral Gene Expression, Journal of Virology 63(9), 3822–3828 (1989).

Beard et al., Transcription Mapping of Mouse Adenovirus Type 1 Early Region 3, Virology 175, 81–90 (1990).

Levrero et al., Defective and nondefective adenovirus vectors for expressing foreign genes in vitro and in vivo, Gene 101, 195–202 (1991).

Graham, Covalently closed circles of humand adenovirus DNA are infectious, The EMBO Journal 3(12), 2917–2922 (1984).

Pinkert et al., An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver–specific expression in transgenic mice, Genes & Development 1, 268–276 (1987).

Adams et al., The c–myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice, Nature 318, 533–538 (1985).

Kelsey et al., Species– and tissue–specific expression of human alpha1–antitrypsin in transgenic mice Genes & Development 1, 161–171 (1987).

Johnson et al., Inhibition of Angiogenesis by Tissue Inhibitor of Metalloproteinase, Journal of Cellular Physiology 160, 194–202 (1994).

Wilhelm et al., Recombinant soluble urokinase receptor as a scavenger for urokinase–type plasminogen activator (uPA) Inhibition of proliferation and invasion of human ovarian cancer cells, FEBS Letters 337, 131–134 (1994).

Hutchinson et al., Mutagenesis at a Specific Position in a DNA Sequence, The Journal of Biological Chemistry 253(18), 6551–6560 (1978).

Oliphant et al., Cloning of random–sequence oligodeoxynucleotides, Gene 44, 177–183 (1986).

Bernstein et al., Gene Transfer with Retrovirus Vectors, Genetic Engineering 7, 235–261 (1985).

McCormick, Human Gene Therapy: The First Round, Bio/Technology 3(8), 689–693 (1985).

Miller et al., Improved Retroviral Vectors for Gene Transfer and Expression, Bio Techniques 7(9), 980–990 (1989).

Felgner et al., Cationic liposome–mediated transfection, Nature 337, 387–388 (1989).

Curiel et al., High–Efficiency Gene Transfer Mediated by Adenovirus Coupled to DNA–Polylysine Complexes, Human Gene Therapy 3, 147–154 (1992).

Zoller et al., Laboratory Methods—Oligonucleotide–Directed Mutagenesis: A Simple Method Using Two Oligonucleotide Primers and a Single–Stranded DNA Template, DNA 3(6), 479–488 (1984).

MacDonald, Expression of the Pancreatic Elastase I Gene in Transgenic Mice, Hepatology 7(1), 42S–51S (1987).

O'Reilly et al., Angiostatin: A Novel Angiogenesis Inhibitor That Mediates the Suppression of Metastases by a Lewis Lung Carcinoma, Cell 79, 315–328 (1994).

Dedieu et al., Long–Term Gene Delivery into the Livers of Immunocompetent Mice with E1/E4–Defective Adenovirus, Journal of Virology 71(6), 4626–4637 (1997).

Mirshahi et al., A monoclonal antibody directed against an epitope in the NH2–terminal region of native human plasminogen induced a modification of its functional properties, Fibrinolysis & Proteolysis 11(3), 155–163 (1997).

Farrell, Recombinant Herpesviruses Lacking Gene For Glycoprotein L, RD#37105A by Cantab Pharmaceuticals Res. Ltd (1995).

Palmer, Gyorffy, et al. "Combined treatment of a murine breast cancer model with type 5 adenovirus vectors expressing murine angiostatin and il–12: a role for combined anit–angiogenesis and immunotherapy," *J. Immunol.,* May 15, 2001, 166(10):6212–7. (Abstract only).

Pili, R., et al. "Adenovirus–mediated gene transfer of fibroblast growth factor–1: angiogenesis and tumorigenicity in nude mice," *Int. J. Cancer,* Oct. 9, 1997, 73(2):258–63. (Abstract only).

Matsumoto, G., et al. "Angiostatin gene therapy inhibits the growth of murine squamous cell carcinoma in vivo," *Oral Oncol,* Jun. 2001, 37(4):369–78. (Abstract only).

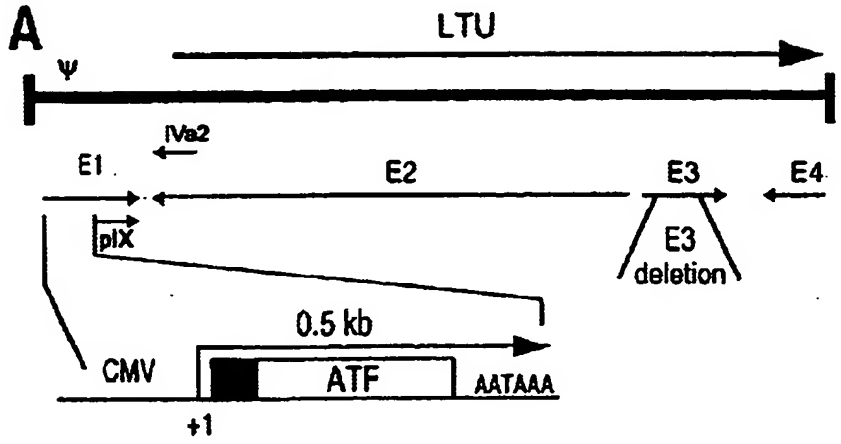
Riccioni, T., et al. "Adenovirus–mediated wild–type p53 overexpression inhibits endothelial cell differentiation in vitro and angiogenesis in vivo," *Gene Ther,* Jun. 1998, 5(6):747–54. (Abstract only).

GenBank entry for human plasminogen and annotations from NCBI web page, Acc. No. P00747.

GenBank entry for human urokinase–type plasminogen activator and annotations from NCBI, Acc. No. P00749.

Abstract #1171 of an article by FUJIWARA et al., titled "Antiangiogenic therapy for human lung cancer by wild–type p53–mediated downregulation of vascular endothelial growth factor (VEGF) expression," Pharmacology/Therapeutics (Preclinical and Clinical) 12, p. 175.

* cited by examiner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E2
E3
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ATF
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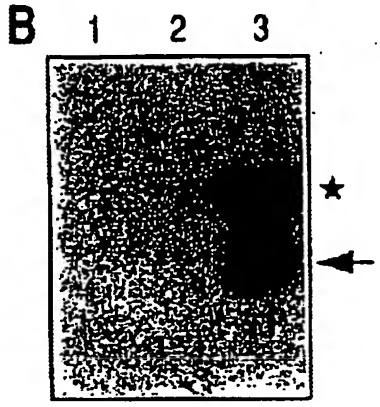
+1


B
1
2
3

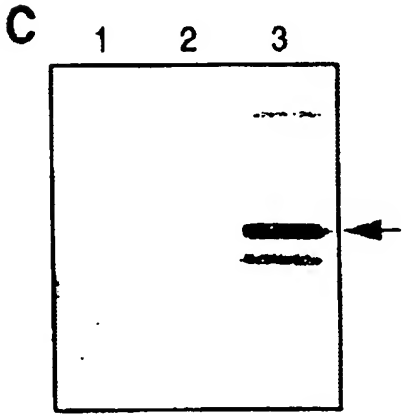
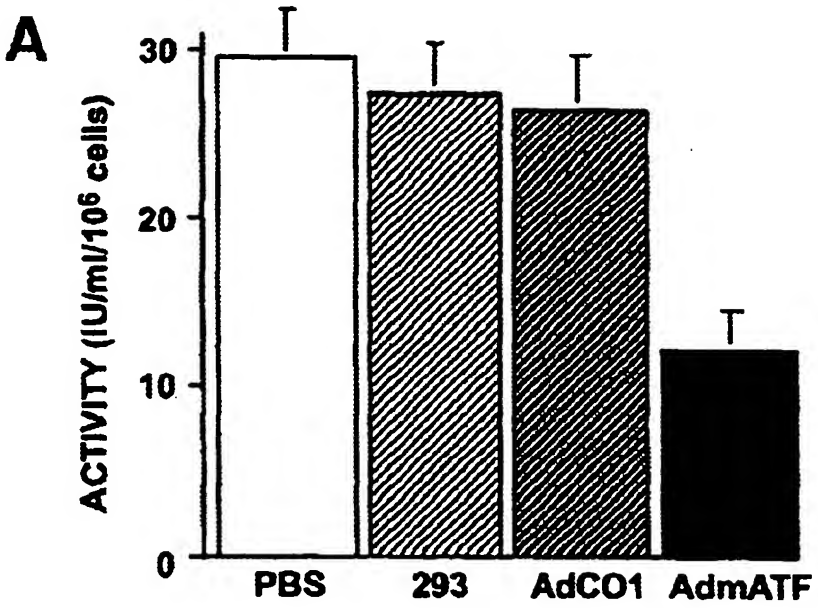

C
1
2
3

FIG. 1



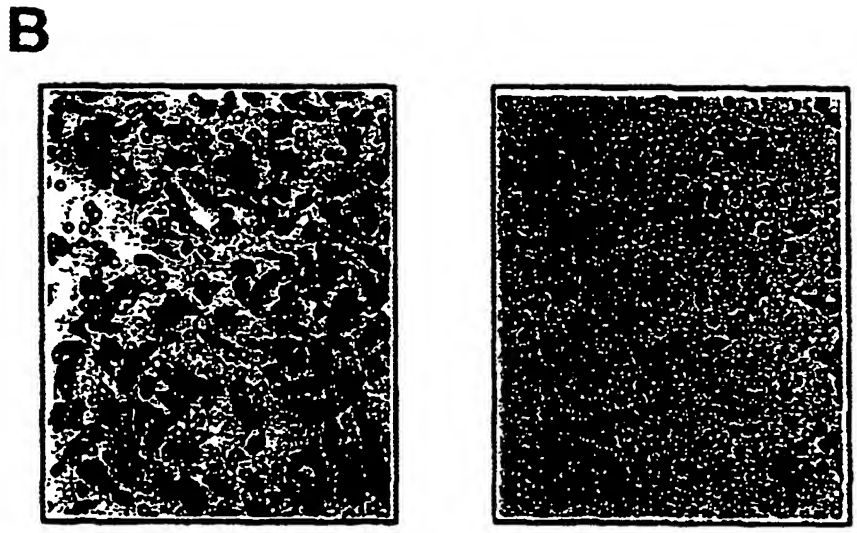


FIG. 2

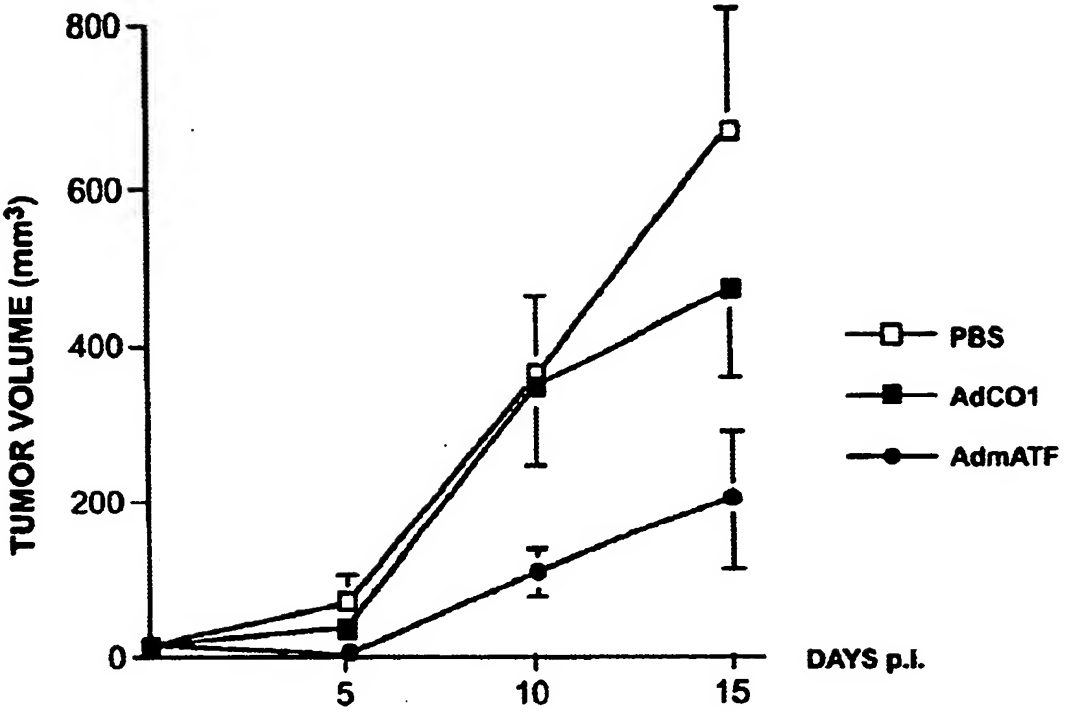

800
600
400
200
0
TUMOR VOLUME (mm³)
5
10
15
DAYS p.i.
PBS
AdCO1
AdmATF

FIG. 3

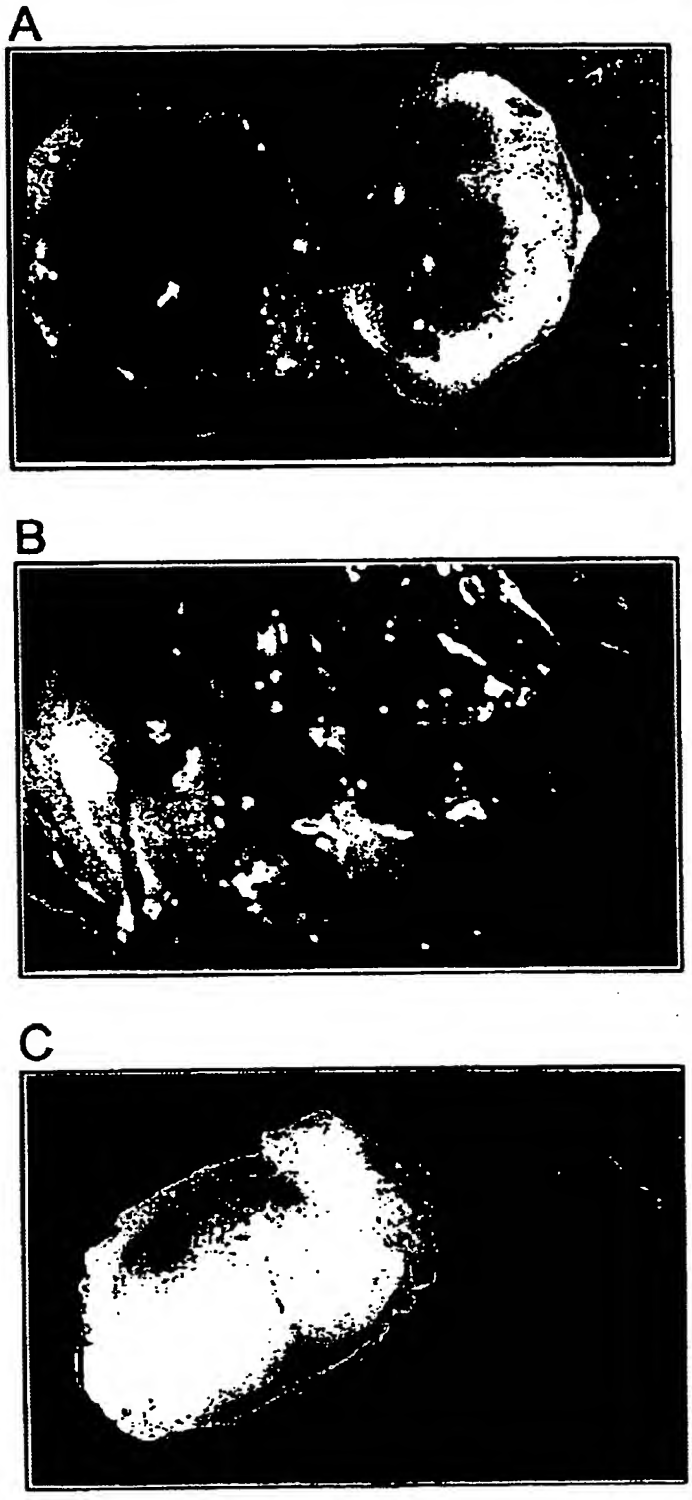


FIG. 4

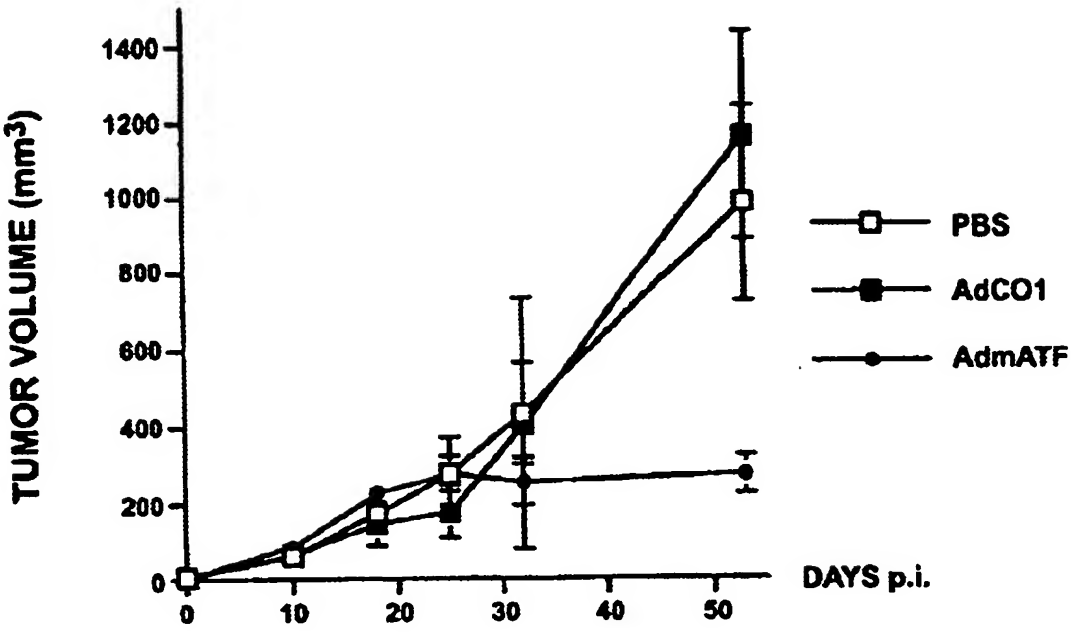

TUMOR VOLUME (mm3)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
DAYS p.i.
PBS
AdCO1
AdmATF

FIG. 5

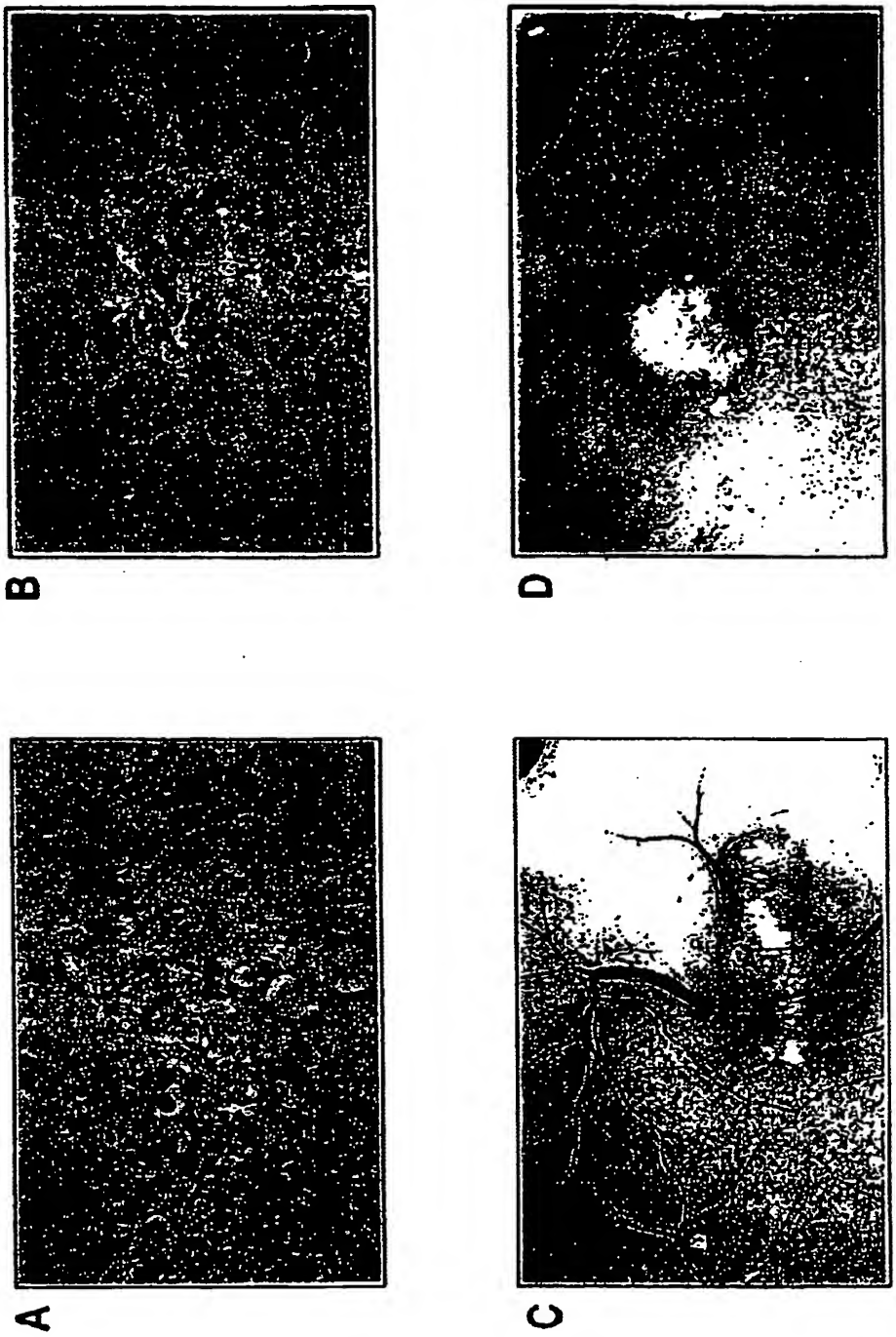
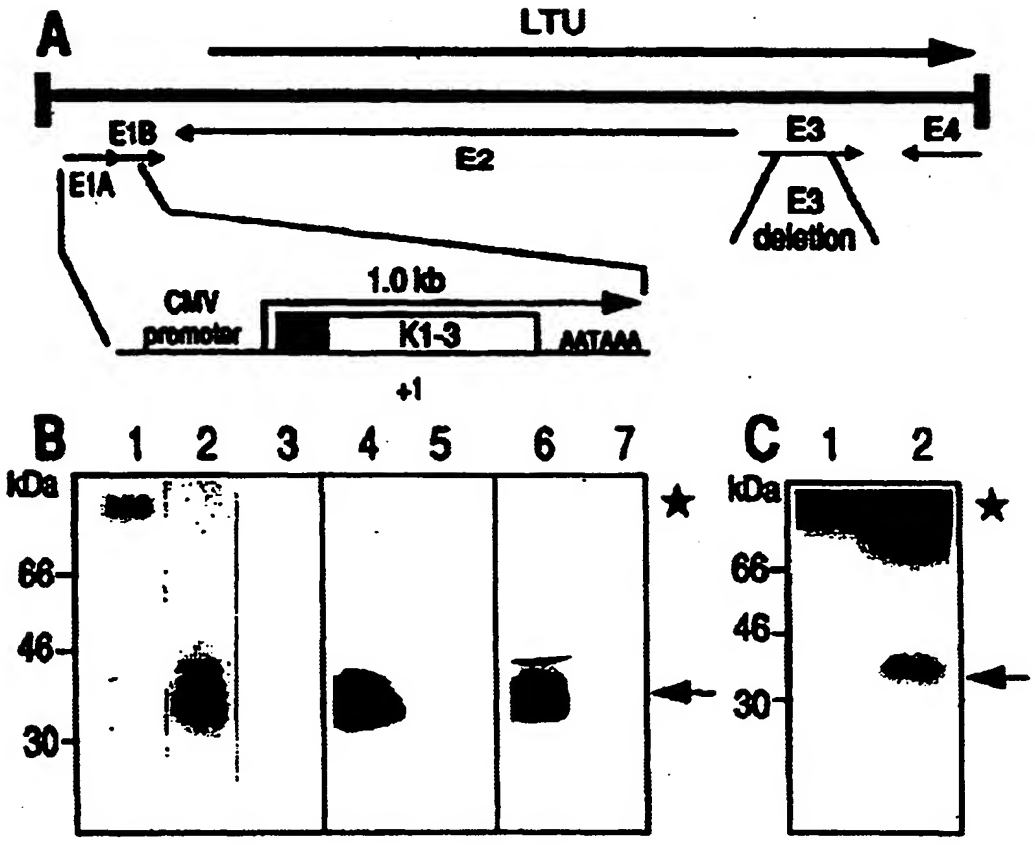


FIG. 6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LTU
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E2
E3
E4
E1A
E3
deletion
1.0 kb
CMV
promoter
K1-3
AATAAA
+1
B
1 2 3 4 5 6 7
kDa
66
46
30
C
1 2
kDa
66
46
30

FIG. 7

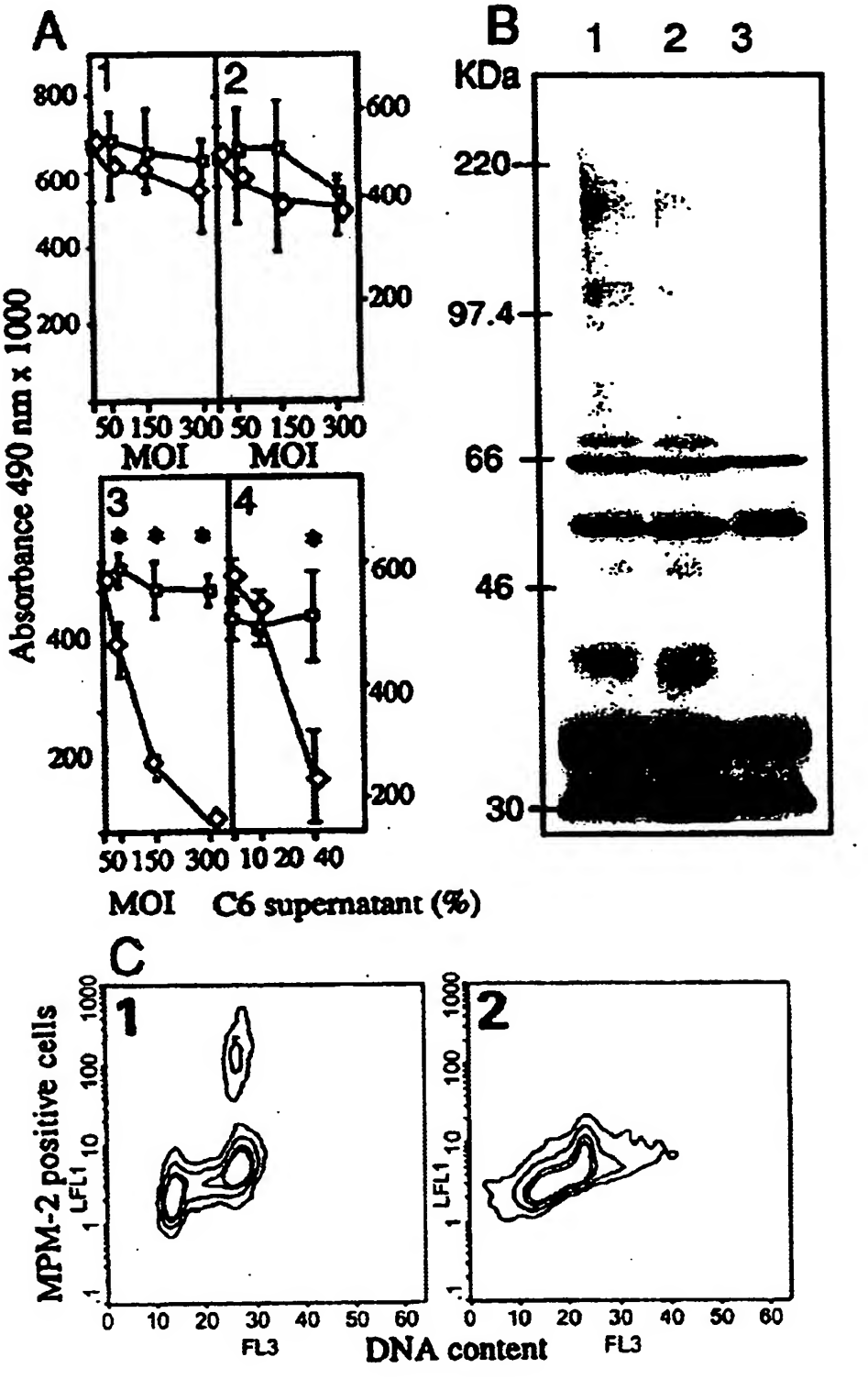
A
B
C
1
2
3
4
Absorbance 490 nm x 1000
MOI
MOI
MOI
C6 supernatant (%)
KDa
220
97.4
66
46
30
MPM-2 positive cells
LFL1
FL3
DNA content

FIG. 8

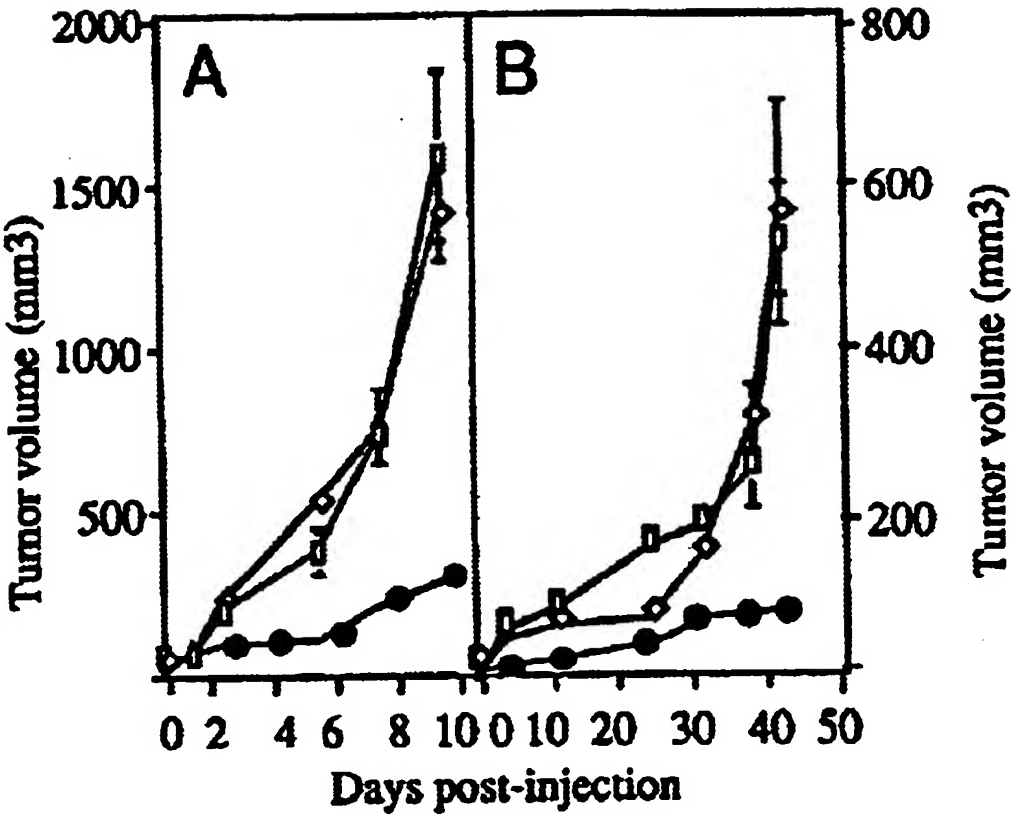
A
B
2000
1500
1000
500
800
600
400
200
Tumor volume (mm3)
Tumor volume (mm3)
0 2 4 6 8 10 0 10 20 30 40 50
Days post-injection

FIG. 9

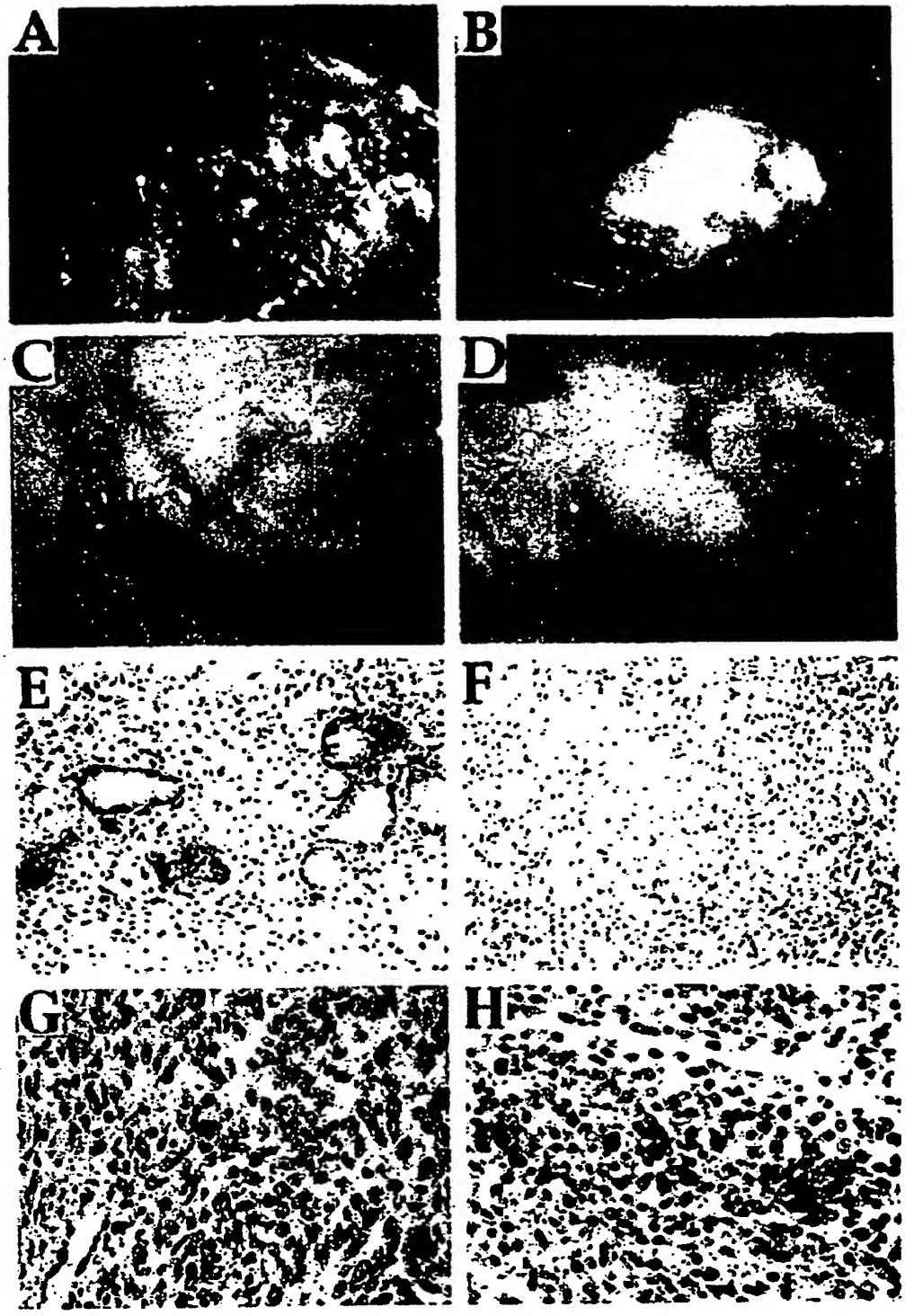

FIG. 10

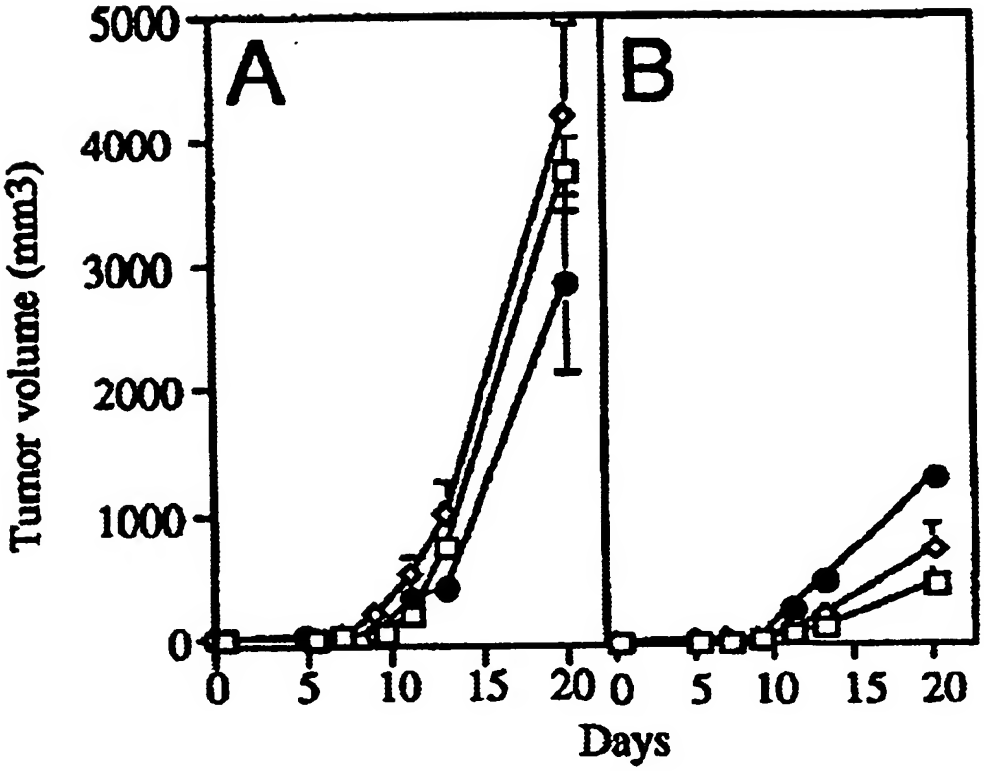
A
B
5000
4000
3000
2000
1000
0
Tumor volume (mm3)
0 5 10 15 20
0 5 10 15 20
Days

FIG. 11

ADENOVIRUS-MEDIATED INTRATUMORAL DELIVERY OF AN ANGIOGENESIS ANTAGONIST FOR THE TREATMENT OF TUMORS

This application claims full benefit of prior applications PCT/EP98/02491 and U.S. provisional application No. 60/044,980 filed on Apr. 28, 1997.

FIELD OF THE INVENTION

The present invention relates to gene therapy for the treatment of tumors. The invention more particularly relates to introduction of a gene encoding an anti-angiogenic factor into cells of a tumor, for example with an adenovirus vector, to inhibit growth or metastasis, or both, of the tumor.

BACKGROUND OF THE INVENTION

Cell migration is a coordinated process that bridges cellular activation and adhesion whereas the equilibrium between pericellular proteolysis and its inhibition (e.g., triggered by plasminogen activator inhibitors and tissue inhibitors of metalloproteinases) is disrupted (1–3). Urokinase plasminogen activator (uPA) is a pivotal player in this process because it initiates a proteolytic cascade at the surface of migrating cells by binding to its cell surface receptor (uPAR) (4, 5). Binding of uPA to its receptor greatly potentiates plasminogen/plasmin conversion at the cell surface (6). Plasmin is a broadly specific serine protease which can directly degrade components of the extracellular matrix such as fibronectin, vitronectin or laminin. Plasmin also indirectly promotes a localized degradation of the stroma by converting inactive zymogens into active metalloproteinases (7). The selective distribution of uPAR at the leading edge of migrating cells (invadopodes) apparently concentrates uPA secreted by themselves or by neighboring stroma cells (8). uPAR is also directly involved in cellular adhesion to the extracellular matrix as illustrated by its uPA-dependent binding to vitronectin (9), and because uPAR modulates the binding properties of several integrin molecules (10). Finally, uPA and plasmin are somehow involved in cell morphogenesis by activating or inducing the release of morphogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factors (FGFs) and transforming growth factor β (TGFβ) (11, 12).

Taken together, these observations indicate that the uPA/uPAR system controls cell migration by coordinating cellular activation, adhesion and motility. This statement is supported by clinical observations that correlate the presence of enhanced uPA activity at the invasive edge of the tumors (13, 14). That melanoma induced by DMBA and croton oil do not progress to a malignant stage in uPA-deficient mice also support a role of uPA in tumor establishment and progression (15).

uPA binds to uPAR by its light chain fragment, also known as amino-terminal fragment (ATF, amino acid 1–135). This interaction is species restricted (16) and involves the EGF-like domain of ATF (residues 1–46), in which amino acid 19–32, which are not conserved between mice and human, are critical (17, 18). ATF-mediated disruption of the uPA/uPAR complex inhibits tumor cell migration and invasion in vitro (19). Intraperitoneal bolus injection of a chimeric human ATF-based antagonist has also been used to inhibit lung metastases of human tumor cells implanted within athymic mice, without significantly affecting primary tumor growth (20). A further study reported that intraperitoneal injection of synthetic peptides derived from murine ATF was effective in inhibiting both primary tumor growth and lung metastases (21). These results are consistent with a role of the uPA/uPAR complex in controlling the motility of both tumor and endothelial cells (22). That a chimeric murine ATF-based antagonist could inhibit vessel growth in an artificial bFGF-enriched extracellular matrix (23) further supports uPA/uPAR involvement in controlling angiogenesis in vivo.

The formation of blood vessels, or angiogenesis, results from the capillary growth of pre-existing vessels. Angiogenesis is essential for a number of physiological processes such as embryonic development, wound healing and tissue or organ regeneration. Abnormal growth of new blood vessels occurs in pathological conditions such as diabetic retinopathy and tumor growth, as well as tumor dissemination to distant sites [38,24]. Both experimental and clinical studies have showed that primary tumors as well as metastasis remain dormant due to a balanced rate of proliferation and apoptosis unless the angiogenesis process is switched on [39].

The growth of endothelial cells is tightly regulated by both positive and negative factors. The onset of tumor angiogenesis could be triggered either by an upregulation of tumor-released angiogenic factors such as vascular endothelial growth factor (VEGF) and acid or/and basic fibroblast growth factor (bFGFs), or by a downregulation of angiostatic factors such as thrombospondin and angiostatin [39]. Both the reconstitution of angiostatic factors and the removal of angiogenic stimulating factors thus constitute plausible clinical strategies to suppress tumor angiogenesis [40, 41]. Angiostatic-based therapies should also apply to all solid tumors because endothelial cells do not vary from one tumor type to the other, further emphasizing the clinical relevance of such an anti-cancer approach. Thus, the therapy targeting angiogenesis appears to be highly relevant to clinical practice.

Many physiological angiostatic factors are derived upon proteolytic cleavage of circulating proteins. This is the case for angiostatin [32], endostatin [42], the 16 kDa fragment of prolactin [43], or platelet factor-4 [44]. Angiostatin was initially isolated from mice bearing a Lewis lung carcinoma (LLC), and was identified as a 38 kDa internal fragment of plasminogen (Plg) (aa 98–440) that encompasses the first four kringles of the molecule [32; WO95/29242; U.S. Pat. No. 5,639,725]. Angiostatin has been shown to be generated following hydrolysis of Plg by a metalloelastase from GM-CSF-stimulated tumor-infiltrating macrophages [45]. Intraperitoneal bolus injections of purified angiostatin in six different tumor models have proved to be very effective in suppressing primary tumor growth, with no apparent toxicity [46]. Angiostatin-mediated suppression of tumor angiogenesis apparently drove the tumor cells into a higher apoptotic rate that counterbalanced their proliferation rate. In this study, tumor growth usually resumed following removal of the angiostatin molecule, emphasizing the importance of achieving long-term delivery for optimal clinical benefits [46]. In vitro studies with recombinant proteins indicated that the angiostatic activity of angiostatin was mostly mediated by kringles 1–3, thus leaving a minor activity for kringle 4 [47]. As for most angiostatic factors, little is known about the molecular pathway by which angiostatin exerts its effect.

As angiostatic therapy will require a prolonged maintenance of therapeutic levels in vivo, the continuous delivery of a recombinant protein will be expensive and cumbersome. Direct in vivo delivery of the corresponding genes

with viral vectors constitutes an attractive solution to this problem. Because most cancer gene therapies currently rely on destructive strategies that target the tumor cells [48], viral-mediated gene delivery of an angiostatic factor represents a conceptually different, and possibly synergistic, approach to fight cancer.

Despite these results, there remains a need to develop effective treatments for tumors, particularly chemotherapy-resistant tumors.

The present invention addresses this need by establishing an effective mode for treating a tumor.

Various references are cited in this specification by number, which are fully set forth after the Examples. None of the references cited herein should be construed as describing or suggesting the invention disclosed herein.

SUMMARY OF THE INVENTION

The present invention advantageously provides a highly effective gene therapy for tumors. Indeed, in a specific embodiment of the invention murine urokinase ATF expressed by human tumor cells in an athymic murine model unexpectedly effectively inhibits tumorigenicity. In another embodiment, angiostatin expressed in tumor cells in a murine model inhibited tumor growth and tumorigenesis, and induced tumor cell apoptosis, in addition to blocking angiogenesis.

In a broad aspect, the present invention provides a method for inhibiting growth or metastasis, or both, of a tumor comprising introducing a vector comprising a gene encoding an anti-angiogenic factor operably associated with an expression control sequence that provides for expression of the anti-angiogenic factor into a cell or cells of the tumor. Preferably, the vector is a virus vector; more preferably the virus vector is an adenovirus vector. In a specific embodiment exemplified infra, the adenovirus vector is a defective adenovirus vector.

The methods of the invention are useful in the treatment of many tumors, as set forth in detail herein. For example, in specific embodiments, the tumor is a lung carcinoma or a breast carcinoma.

In addition, the invention demonstrates for the first time the advantages of expression of an anti-angiogenic factor by the transduced tumor cells. Accordingly, a gene encoding any anti-angiogenic factor, such as a soluble receptor for an angiogenic protein, or an angiogenesis antagonist, can be delivered in the practice of the invention. In a specific embodiment, the anti-angiogenic factor comprises a sequence of an amino terminal fragment of urokinase having an EGF-like domain, with the proviso that the factor is not urokinase. For example, the anti-angiogenic factor may be a chimeric protein comprising ATF-immunoglobulin or ATF-human serum albumin. In a preferred embodiment, exemplified infra, the anti-angiogenic factor is an amino terminal fragment of urokinase having an amino acid sequence of urokinase from about amino acid residue 1 to about residue 135. In a specific aspect, the urokinase is murine urokinase. In a more preferred aspect, the urokinase is human urokinase.

In an alternative embodiment, the anti-angiogenic factor is angiostatin, in particular, kringles 1 to 3 of angiostatin. In a particularly preferred embodiment, the anti-angiogenic factor is the amino-terminal fragment of plasminogen (Plg) having an amino acid sequence of plasminogen from about amino acid residue 1 to about residue 333. In another preferred embodiment, the anti-angiogenic factor is the amino-terminal fragment (angiostatin) from human plasminogen.

In a related embodiment, the invention is directed to use of a vector comprising a gene encoding an anti-angiogenic factor operably associated with an expression control sequence that provides for expression of the anti-angiogenic factor in the manufacture of a medicament for inhibiting growth or metastasis, or both, of a tumor. More particularly, the invention provides for use of a virus vector of the invention, e.g., as set out below, in the manufacture of a medicament for inhibiting growth or metastasis, or both, of a tumor.

Naturally, in addition to the foregoing methods and uses, the invention provides a novel virus vector comprising a gene encoding an anti-angiogenic factor operably associated with an expression control sequence. In a preferred embodiment, the virus vector is an adenovirus vector. In a more preferred embodiment, the virus vector is a defective adenovirus vector. The virus vectors of the invention can provide a gene encoding any anti-angiogenic factor, as set forth above. For example, the anti-angiogenic factor may comprise a sequence of an amino terminal fragment of urokinase having an EGF-like domain, with the proviso that the factor is not urokinase. In a preferred embodiment, the anti-angiogenic factor is an amino terminal fragment of urokinase having an amino acid sequence of urokinase from amino acid residue 1 to about residue 135. In this embodiment, the urokinase may be murine urokinase or, preferably, human urokinase.

The invention further provides a pharmaceutical composition any of the virus vectors of the invention and a pharmaceutically acceptable carrier.

Thus, one object of the invention is to provide gene therapy by delivery of anti-angiogenic factors for treating tumors.

Another object of the invention is to provide a viral vector for delivery of an anti-tumorigenic factor.

Still another object of the invention is to provide an amino terminal fragment of urokinase (ATF) by gene therapy for treatment of a tumor.

Further, another object of the invention is to provide angiostatin by gene therapy for treatment of a tumor.

Yet another object of the invention is to provide angiostatin, particularly kringles 1 to 3 of angiostatin, by gene therapy for treatment of a tumor.

These and other objects of the invention are further elaborated in the following Detailed Description and Examples, and the accompanying drawings.

DESCRIPTION OF THE DRAWINGS

FIG. 1. Molecular characterization of virus AdmATF. Panel A: Structure of AdmATF and AdCO1. The Ad5 chromosome is 36 kb long and bordered by inverted terminal repeats. Y refers to the encapsidation signal. Both viruses are defective for growth because they lack the Ad5 E1 genes. They also carry a 1.9 kb XbaI deletion within region E3. A schematic representation of the mATF expression cassette of virus AdmATF is indicated under the Ad5 chromosome (not drawn to scale). For a review on adenoviral vectors see (38). Panel B: analysis of mATF expression. MDA-MB-231 cells were infected for 24 hr by AdCO1 (lane 2) or AdmATF (lane 3), or mock-infected (lane 1), and total poly(A+) RNAs were submitted to northern blot analysis. The ATF-encoding RNA (0.5 kb) is indicated (arrow). A 1.7 kb molecule is also detected (asterisk), a size in agreement with the utilization of the polyadenylation signal from the adenovirus pIX gene. Panel C: analysis of ATF secretion by 293-infected cells.

The culture media of mock-infected cells (lane 1), or infected with AdCO1 (lane 2) or AdmATF (lane 3) were submitted to a western blot analysis with a polyclonal anti-mouse uPA antibody.

FIG. 2. Functional characterization of virus AdmATF. Panel A: The culture medium of AdmATF-infected cells inhibits plasmin conversion at the surface of LLC cells (see Methods section of the Example). 293 refers to the supernatant of non infected cells. Panel B: Infection of LLC cells with AdmATF (right panel) specifically inhibits cell invasiveness as compared to that of LLC cells infected with AdCO1 (left panel). The 1.2 mm pores of the membranes are visible.

FIG. 3. Intratumoral injection of AdmATF inhibits LLC tumor growth in syngeneic mice. Tumor cells (2×10^6 cells) were subcutaneously injected into C57BL/6 mice. After 6 days, the animals received an intratumoral injection of PBS, or 10^9 PFU of AdCO1 or AdmATF and tumor growth was monitored. The mean values are represented with their standard variations (n=10). Statistics were done with the Student test.

FIG. 4. Intratumoral injection of AdmATF inhibits LLC tumor vascularization. Panel A: a representative tumor from the AdCO1-treated (left) and AdmATF-treated groups extracted at day 10 p.i. is shown. A representative tumor extracted at day 20 p.i. is shown in panel B (injection with AdCO1) and panel C (injection with AdmATF). All photographs were taken at the same magnification. Note that the AdmATF-injected tumors are much smaller that their AdCO1-injected controls, especially at the latest time p.i. (compare panels B and C).

FIG. 5. Intratumoral injection of AdmATF inhibits MDA-MB-231 tumor growth in nude mice. Tumors were implanted by subcutaneous injection of 3×10^6 MDA-MB-231 cells. At day 11 post implantation, the mice received an intratumoral injection of PBS, or 10^9 PFU of AdmATF or AdCO1, and the tumor growth was monitored. The mean values are represented with their standard variations.

FIG. 6. Intratumoral injection of AdmATF inhibits intratumoral and peritumoral angiogenesis. Panels A and B: vWF immunostaining of tumor sections. Paraffin embedded MDA-MB-231 tumor sections prepared from the AdCO1-(A) and AdmATF-treated groups (B) were revealed with a polyclonal anti-vWF serum at day 52 p.i. Panels C and D: Macroscopic evaluation of peritumoral vascularization within the skin of tumors injected with AdCO1 (C) or AdmATF (D) at day 20 p.i.

FIG. 7. (A) Recombinant adenoviruses. The AdS genome is a 36 kb-long chromosome. Viruses AdK3 and AdCO1 were derived by a lethal deletion of the E1 genes (nucleotide position 382 to 3446); they also carry a non-lethal 1.9 kb XbaI deletion within region E3 (for a review see [37]). The angiostatin expression cassette is shown under the Ad5 chromosome. The plasminogen secretion signal is represented by a blackened box; +1 refers to the CMV-driven transcription start; AATAAA refers to the SV40 late polyadenylation signal. (B) Analysis of angiostatin secretion from infected-cells. 100 ng of human Plg (lane 1), culture medium from HMEC-1 infected with AdK3 (lane 2) or AdCO1 (lane 3), C6 infected with AdK3 (lane 4) or AdCO1 (lane 5), and from MDA-MB-231 infected with AdK3 (lane 6) or AdCO1 (lane 7) were submitted to Western blot analysis. (C) Immuno-detection of angiostatin within C6 tumor extracts; Tumors were established in nude mice and received 10^9 PFU of AdCO1 (lane 1) or AdK3 (lane 2) and Western blot analysis was performed 10 days p.i. The signal corresponding to angiostatin (36–38 kDa) and Plg (92 kDa) are indicated (arrow and asterisk respectively).

FIG. 8. (A) Inhibition of endothelial cell proliferation. C6 (panel 1), MDA-MB-231 (panel 2) and HMEC-1 (panel 3) were injected with AdK3 (♦) or Ad-CO1 (□). HMEC-1 cells (panel 4) cultured with the supernatant from AdK3-(♦) or AdCO1-infected C6 glioma cells (□). (B) Detection of MPM-2 phosphoepitope in HMEC-1 cells. Mock-infected cells (lane 1), AdCO1-infected cells (lane 2), and AdK3-infected cells (lane 3). (C) MPM-2 epitope were detected in HMEC-1 infected with AdCO1 (panel 1) or AdK3 (panel 2) by indirect immunostaining and DNA content by propidium iodide staining, and quantified by flow cytometry (see Methods). A Student's t-test was used for statistical analysis.

FIG. 9. AdK3 inhibits tumor growth. C6 glioma (panel A) and MDA-MB-231 carcinoma (panel B) were subcutaneously implanted into athymic mice (see Methods). When the tumor had reached a volume of 20 mm^3 (day 0), mice received an intratumoral injection of PBS (□), or 10^9 PFU or AdK3 (●) or AdCO1 (♦). Mean values are represented with their standard deviations.

FIG. 10. AdK3 inhibits C6 tumor growth and angiogenesis. Tumors from AdCO1-treated (panel A) and AdK3-treated groups (panel B) are shown 10 days p.i. The extent of vascularization at the periphery of a representative tumor injected with AdCO1 (panel C) or AdK3 (panel D) is shown at day 5 p.i. Paraffin-embedded C6 sections from an AdCO1-injected (panel E) or an AdK3-injected tumor (panel F) were submitted to vWF-immunostaining at day 10 p.i. The proportion of apoptotic cells was detected by the TUNEL method within sections from an AdCO1-injected (panel G) or an AdK3-injected tumor (panel H). The same magnification was used for AdCO1- and AdK3-injected tumors.

FIG. 11. Dose dependent effect of AdK3. C6 cells were infected in vitro, 24 hours with AdCO1 (panel A) or Ad3 K (panel B) and mixed with a ratio of 1 (□), 1:2 (♦) and 1:4 (●) with non-infected C6 cells, prior to C6 cells implantation into athymic mice. Tumor volumes were measured during 20 days. Mean values are represented with their standard deviations.

DETAILED DESCRIPTION OF THE INVENTION

As disclosed above, the present invention is directed to methods and vectors for gene therapy of tumors. The methods and vectors of the invention inhibit tumor growth or tumor metastasis, or both. These methods and vectors act by inhibiting angiogenesis of the tumor to an unexpectedly advantageous degree.

The invention is based, in part, on experiments involving gene therapy delivery of the amino terminal fragment of urokinase (ATF) and angiostatin. ATF is an antagonist of urokinase (uPA) binding to its cell surface receptor (uPAR), and an inhibitor of endothelial cell migration. To assess the importance of the uPA/uPAR interaction for tumor growth and dissemination, a defective adenovirus expressing murine ATF from the CMV promoter (AdmATF) was constructed. A single intratumoral injection of AdmATF inhibited growth of pre-established tumors in two different murine models, and delayed tumor dissemination. These effects were correlated with a remarkable inhibition of neovascularization within, and at the immediate vicinity of, the injection site. The magnitude of this effect was particularly remarkable in the ability of murine ATF to inhibit angiogenesis of a human-derived tumor. In a specific example, a defective adenovirus that expresses the

N-terminal fragment (aa 1-333) from human Plg, including the pre-activation peptide and kringles 1 to 3 [47] was constructed (AdK3) and its in vitro and in vivo activity in different murine tumor models was assessed. The AdK3 vector inhibited tumor growth, tumor angiogenesis, and tumorigenesis, and induced tumor cell apoptosis.

Intratumoral adenovirus-mediated delivery of antagonist displays potent antitumoral properties by targeting angiogenesis.

Definitions

The following defined terms are used throughout the present specification, and should be helpful in understanding the scope and practice of the present invention.

In a specific embodiment, the term "about" or "approximately" means within. 20%, preferably within 10%, and more preferably within 5% of a given value or range.

An "anti-angiogenic" factor is a molecule that inhibits angiogenesis, particularly by blocking endothelial cell migration. Such factors include fragments of angiogenic proteins that are inhibitory (such as the ATF of urokinase), angiogenesis inhibitory factors, such as angiostatin and endostatin; and soluble receptors of angiogenic factors, such as the urokinase receptor or FGF/VEGF receptor. The term "angiostatin", which is derived from the amino-terminal fragment of plasinogen, includes the anti-angiogenic fragment of angiostatin having kringles 1 to 3. Generally, an anti-angiogenic factor for use in the invention is a protein or polypeptide encoded by a gene transfected into tumors using the vectors of the invention.

A "variant" of a polypeptide or protein is any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements. These variants may include, inter alia: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the polypeptide or protein, (c) variants in which one or more of the amino acids includes a substituent group, and (d) variants in which the polypeptide or protein is fused with another polypeptide such as serum albumin. The techniques for obtaining these variants, including genetic (suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques, are known to persons having ordinary skill in the art.

If such allelic variations, analogues, fragments, derivatives, mutants, and modifications, including alternative mRNA splicing forms and alternative post-translational modification forms result in derivatives of the polypeptide which retain any of the biological properties of the polypeptide, they are intended to be included within the scope of this invention.

General Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D. N. Glover ed. 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. 1984); *Nucleic Acid Hybridization* [B. D. Hames & S. J. Higgins eds. (1985)]; *Transcription And Translation* [B. D. Hames & S. J. Higgins, eds. (1984)]; *Animal Cell Culture* [R. I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F. M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is any means for the transfer of a nucleic acid according to the invention into a host cell. The term "vector" includes both viral and nonviral means for introducing the nucleic acid into a cell in vitro, ex vivo or in vivo. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers. Viral vectors include retrovirus, adeno-associated virus, pox, baculovirus, vaccinia, herpes simplex, Epstein-Barr and adenovirus vectors, as set forth in greater detail below. In addition to a nucleic acid according to the invention, a vector may also contain one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues, duration of expression, etc.).

"Regulatory region" means a nucleic acid sequence which regulates the expression of a second nucleic acid sequence. A regulatory region may include sequences which are naturally responsible for expressing a particular nucleic acid (a homologous region) or may include sequences of a different origin (responsible for expressing different proteins or even synthetic proteins). In particular, the sequences can be sequences of eukaryotic or viral genes or derived sequences which stimulate or repress transcription of a gene in a specific or non-specific manner and in an inducible or non-inducible manner. Regulatory regions include origins of replication, RNA splice sites, enhancers, transcriptional termination sequences, signal sequences which direct the polypeptide into the secretory pathways of the target cell, and promoters.

A regulatory region from a "heterologous source" is a regulatory region which is not naturally associated with the expressed nucleic acid. Included among the heterologous regulatory regions are regulatory regions from a different species, regulatory regions from a different gene, hybrid regulatory sequences, and regulatory sequences which do not occur in nature, but which are designed by one having ordinary skill in the art.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" or "transduced" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

A "nucleic acid" is a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA, genomic DNA, synthetic DNA, and semi-synthetic DNA. The sequence of nucleotides or nucleic acid sequence that encodes a protein is called the sense sequence. A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then optionally trans-RNA spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

The various aspects of the invention will be set forth in greater detail in the following sections, directed to suitable gene therapy vectors and promoters, anti-angiogenic proteins, and therapeutic strategies. This organization into various sections is intended to facilitate understanding of the invention, and is in no way intended to be limiting thereof.

Gene Therapy Vectors

As discussed above, a "vector" is any means for the transfer of a nucleic acid according to the invention into a host cell. Preferred vectors are viral vectors, such as retroviruses, herpes viruses, adenoviruses and adeno-associated viruses. Thus, a gene or nucleic acid sequence encoding an anti-angiogenic protein or polypeptide domain fragment thereof is introduced in vivo, ex vivo, or in vitro using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both.

Viral vectors commonly used for in vivo or ex vivo targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art [see, e.g., Miller and Rosman, *BioTechniques* 7:980–990 (1992)]. Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed in vitro (on the isolated DNA) or in situ, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably, the replication defective virus retains the sequences of its genome which are necessary for encapsulating the viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein-Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt et al., *Molec. Cell. Neurosci.* 2:320–330 (1991)], defective herpes virus vector lacking a glyco-protein L gene [Patent Publication RD 371005 A], or other defective herpes virus vectors [International Patent Publication No. WO 94/21807, published Sep. 29, 1994; International Patent Publication No. WO 92/05263, published Apr. 2, 1994]; an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. [*J. Clin. Invest.* 90:626–630 (1992); see also La Salle et al., *Science* 259:988–990 (1993)]; and a defective adeno-associated virus vector [Samulski et al., *J. Virol.* 61:3096–3101 (1987); Samulski et al., *J. Virol.* 63:3822–3828 (1989); Lebkowski et al., *Mol. Cell. Biol.* 8:3988–3996 (1988)].

Preferably, for in vivo administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector, e.g., adenovirus vector, to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-γ(IFN-γ), or anti-CD4

antibody, can be administered to block humoral or cellular immune responses to the viral vectors [see, e.g., Wilson, *Nature Medicine* (1995)]. In addition, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

Adenovirus vectors. In a preferred embodiment, the vector is an adenovirus vector. As shown in the Examples, defective adenovirus vectors have shown themselves to be particularly effective for delivery of the angiogenesis inhibitors ATF and angiostatin, as shown by the unexpectedly efficient effects of inhibiting tumor growth and tumorigenesis. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g., Manhattan or A26/61 strain (ATCC VR-800), for example).

Preferably, the replication defective adenoviral vectors of the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/28938), the E4 region (WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1–L5.

In a preferred embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185,573, the contents of which are incorporated herein by reference. In another preferred embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378, the contents of which are incorporated herein by reference. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted (see FR94 13355, the contents of which are incorporated herein by reference).

The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, inter alia, the DNA sequence of interest. The homologous recombination is effected following cotransfection of the said adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably (i) be transformable by the said elements, and (ii) contain the sequences which are able to complement the part of the genome of the replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used are the human embryonic kidney cell line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines which are able to complement the E1 and E4 functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Adeno-associated viruses. The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions which carry the encapsidation functions: the left-hand part of the genome, which contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, which contains the cap gene encoding the capsid proteins of the virus.

The use of vectors derived from the AAVs for transferring genes in vitro and in vivo has been described (see WO 91/18088; WO 93/09239; U.S. Pat. No. 4,797,368, U.S. Pat. No. 5,139,941, EP 488 528). These publications describe various AAV-derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the said gene of interest in vitro (into cultured cells) or in vivo, (directly into an organism). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV recombinants which are produced are then purified by standard techniques.

The invention also relates, therefore, to an AAV-derived recombinant virus whose genome encompasses a sequence encoding a nucleic acid encoding an anti-angiogenic factor flanked by the AAV ITRs. The invention also relates to a plasmid encompassing a sequence encoding a nucleic acid encoding an anti-angiogenic factor flanked by two ITRs from an AAV. Such a plasmid can be used as it is for transferring the nucleic acid sequence, with the plasmid, where appropriate, being incorporated into a liposomal vector (pseudo-virus).

Retrovirus vectors. In another embodiment the gene can be introduced in a retroviral vector, e.g., as described in Anderson et al., U.S. Pat. No. 5,399,346; Mann et al., 1983, Cell 33:153; Temin et al., U.S. Pat. No. 4,650,764; Temin et al., U.S. Pat. No. 4,980,289; Markowitz et al., 1988, J. Virol. 62:1120; Temin et al., U.S. Pat. No. 5,124,263; EP 453242, EP178220; Bernstein et al. Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689; International Patent Publication No. WO 95/07358, published Mar. 16, 1995, by Dougherty et al.; and Kuo et al., 1993, Blood 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the gag, pol and env genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of

interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are disclosed in WO95/02697.

In general, in order to construct recombinant retroviruses containing a nucleic acid sequence, a plasmid is constructed which contains the LTRs, the encapsidation sequence and the coding sequence. This construct is used to transfect a packaging cell line, which cell line is able to supply in trans the retroviral functions which are deficient in the plasmid. In general, the packaging cell lines are thus able to express the gag, pol and env genes. Such packaging cell lines have been described in the prior art, in particular the cell line PA317 (U.S. Pat. No. 4,861,719); the PsiCRIP cell line (WO90/02806) and the GP+envAm-12 cell line (WO89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences which may include a part of the gag gene (Bender et al., J. Virol. 61 (1987) 1639). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

Retroviral vectors can be constructed to function as infectious particles or to undergo a single round of transfection. In the former case, the virus is modified to retain all of its genes except for those responsible for oncogenic transformation properties, and to express the heterologous gene. Non-infectious viral vectors are prepared to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus.

Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Non-viral Vectors. Alternatively, the vector can be introduced in vivo as nucleic acid free of transfecting excipients, or with transfection facilitating agents, e.g., lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids in vitro. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for in vivo transfection of a gene encoding a marker [Feigner, et. al., *Proc. Natl. Acad. Sci. U.S.A.* 84:7413–7417 (1987); see Mackey, et al., *Proc. Natl Acad. Sci. U.S.A.* 85:8027–8031 (1988); Ulmer et al., *Science* 259:1745–1748 (1993)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Feigner and Ringold, *Science* 337:387–388 (1989)]. Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Pat. No. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs in vivo has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting [see Mackey, et. al., supra]. Targeted peptides, e.g., hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid in vivo, such as a cationic oligopeptide (e.g., International Patent Publication WO95/2193 1), peptides derived from DNA binding proteins (e.g., International Patent Publication WO96/25508), or a cationic polymer (e.g., International Patent Publication WO95/21931).

It is also possible to introduce the vector in vivo as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter [see, e.g., Wu et al., *J. Biol. Chem.* 267:963–967 (1992); Wu and Wu, *J. Biol. Chem.* 263:14621–14624 (1988); Hartmut et al., Canadian Patent Application No. 2,012,311, filed Mar. 15, 1990; Williams et al., *Proc. Natl. Acad. Sci. USA* 88:2726–2730 (1991)]. Receptor-mediated DNA delivery approaches can also be sued [Curiel et al., *Hum. Gene Ther.* 3:147–154(1992); Wu and Wu, *J Biol. Chem.* 262:4429–4432 (1987)].

The nucleic acid can also be administered as a naked DNA. Methods for formulating and administering naked DNA to mammalian muscle tissue are disclosed in U.S. Pat. No. 5,580,859 and 5,589,466, the contents of which are incorporated herein by reference.

Regulatory Regions. Expression of an anti-angiogenic factor from a vector of the invention may be controlled by any regulatory region, i.e., promoter/enhancer element known in the art, but these regulatory elements must be functional in the host target tumor selected for expression.

The regulatory regions may comprise a promoter region for functional transcription in the tumor, as well as a region situated in 3' of the gene of interest, and which specifies a signal for termination of transcription and a polyadenylation site. All these elements constitute an expression cassette.

Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoter may be naturally responsible for the expression of the nucleic acid. It may also be from a heterologous source. In particular, it may be promoter sequences of eukaryotic or viral genes. For example, it may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, it may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this regard, there may be mentioned, for example, the promoters of the E1A, MLP, CMV and RSV genes and the like.

In addition, the promoter may be modified by addition of activating or regulatory sequences or sequences allowing a tissue-specific or predominant expression (enolase and GFAP promoters and the like). Moreover, when the nucleic acid does not contain promoter sequences, it may be inserted, such as into the virus genome downstream of such a sequence.

Some promoters useful for practice of this invention are ubiquitous promoters (e.g., HPRT, vimentin, actin, tubulin), intermediate filament promoters (e.g., desmin, neurofilaments, keratin, GFAP), therapeutic gene promoters (e.g., MDR type, CFTR, factor VIII), tissue-specific promoters (e.g., actin promoter in smooth muscle cells), promoters which are preferentially activated in dividing cells, promoters which respond to a stimulus (e.g., steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Tetracycline-regulated transcriptional modula-

tors and CMV promoters are described in WO 96/01313, U.S. Pat. Nos. 5,168,062 and 5,385,839, the contents of which are incorporated herein by reference.

Thus, the promoters which may be used to control gene expression include, but are not limited to, the cytomegalovirus (CMV) promoter, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304–310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787–797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441–1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39–42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727–3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21–25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74–94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639–646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399–409; MacDonald, 1987, Hepatology 7:425–515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115–122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647–658; Adames et al., 1985, Nature 318:533–538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436–1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485–495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268–276), alpha-fetoprotein gene control region which is active in liver (Krumlaufet al., 1985, Mol. Cell. Biol. 5:1639–1648; Hammer et al., 1987, Science 235:53–58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161–171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338–340; Kollias et al., 1986, Cell 46:89–94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703–712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283–286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372–1378).

Genes Encoding Anti-Angiogenic Proteins

The vectors of the invention can be used to deliver a gene encoding an anti-angiogenic protein into a tumor in accordance with the invention. In a preferred embodiment, the anti-angiogenic factor is the amino terminal fragment (ATF) of urokinase, containing the EGF-like domain. Such fragment corresponds to amino acid residues about 1 to about 135 of ATF.

In another embodiment, ATF may be provided as a fusion protein, e.g., with immunoglobulin or human serum albumin [WO93/15199], which is specifically incorporated herein by reference in its entirety.

An effective ATF for use in the invention can be derived from any urokinase, such as murine urokinase, although human urokinase ATF is preferred. In addition, the invention contemplates administration of a non-human urokinase ATF modified by substitution of specific amino acid residues with the corresponding residues from human ATF. For example, murine ATF can be modified at one or more, and preferably all, amino acid residues as follows: tyrosine-23 to asparagine; arginine-28 to asparagine; arginine-30 to histidine; and arginine-31 to tryptophan. Thus, urokinase ATF from any source can be humanized. This is easily accomplished by modifying the coding sequence using routine molecular biological techniques.

Genes encoding other anti-angiogenesis protein can also be used according to the invention. Such genes include, but are not limited to, genes encoding angiostatin [O'Reilly et al., Cell 79:315–328 (1994); WO95/29242; U.S. Pat. No. 5,639,725], including angiostatin comprising kringles 1 to 3; tissue inhibition of metalloproteinase [Johnson et al., J. Cell. Physiol. 160:194–202 (1994)]; inhibitors of FGF or VEGF; and endostatin [WO97/15666]. In a preferred embodiment, the anti-angiogenic factor is angiostatin, particularly kringles 1 to 3 of angiostatin. In a particularly preferred embodiment, the anti-angiogenic factor is the amino-terminal fragment of plasminogen (Plg) having an amino acid sequence of plasminogen from about amino acid residue 1 to about residue 333. In another preferred embodiment, the amino terminal fragment of plasminogen/angiostatin is human plasminogen (angiostatin).

In another embodiment, the invention provides for administration of genes encoding soluble forms of receptors for angiogenic factors, including but not limited to soluble VGF/VEGF receptor, and soluble urokinase receptor [Wilhem et al., FEBS Letters 337:131–134 (1994)].

In general, any gene encoding a protein or soluble receptor that antagonizes endothelial cell activation and migration, which is involved in angiogenesis, can be employed in the gene therapy vectors and methods of the invention. Notwithstanding, it is particularly noteworthy that gene therapy delivery of ATF or angiostatin is especially effective in this regard, for reasons pointed out above and exemplified below.

A gene encoding an anti-angiogenic factor, whether genomic DNA or cDNA, can be isolated from any source, particularly from a human cDNA or genomic library. Methods for obtaining such genes are well known in the art, as described above [see, e.g., Sambrook et al., 1989, supra].

Due to the degeneracy of nucleotide coding sequences, other nucleic acid sequences which encode substantially the same amino acid sequence as an anti-angiogenic factor gene may be used in the practice of the present invention and these are contemplated as falling within the scope of the claimed invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of anti-angiogenic factor genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the anti-angiogenic factor derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of an anti-angiogenic factor protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent,

resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

Lys for Arg and vice versa such that a positive charge may be maintained;

Glu for Asp and vice versa such that a negative charge may be maintained;

Ser for Thr such that a free —OH can be maintained; and

Gln for Asn such that a free $CONH_2$ can be maintained.

The genes encoding anti-angiogenic factor derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned anti-angiogenic factor gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, supra). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated in vitro. In the production of the gene encoding a derivative or analog of anti-angiogenic factor, care should be taken to ensure that the modified gene remains within the same translational reading frame as the anti-angiogenic factor gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the anti-angiogenic factor-encoding nucleic acid sequence can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further in vitro modification, such as to form a chimeric gene. Preferably, such mutations enhance the functional activity of the mutated anti-angiogenic factor gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479–488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61–70).

Therapeutic Targets and Strategies

The process according to the present invention enables one to treat tumors. According to the present invention, it is now possible, by ajudicious choice of various injections, infusions, direct application, etc., to infect specifically and unilaterally a large number of tumor cells.

Pharmaceutical Compositions. For their use according to the present invention, the vectors, either in the form of a virus vector, nucleic acid-lipid composition, or naked DNA, are preferably combined with one or more pharmaceutically acceptable carriers for an injectable formulation. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, allow the constitution of injectable solutions.

The preferred sterile injectable preparations can be a solution or suspension in a nontoxic parenterally acceptable solvent or diluent. Examples of pharmaceutically acceptable carriers are saline, buffered saline, isotonic saline (e.g., monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, or mixtures of such salts), Ringer's solution, dextrose, water, sterile water, glycerol, ethanol, and combinations thereof. 1,3-butanediol and sterile fixed oils are conveniently employed as solvents or suspending media. Any bland fixed oil can be employed including synthetic mono- or di-glycerides. Fatty acids such as oleic acid also find use in the preparation of injectables.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

The virus doses used for the administration may be adapted as a function of various parameters, and in particular as a function of the site (tumor) of administration considered, the number of injections, the gene to be expressed or alternatively the desired duration of treatment. In general, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu, and preferably 10^6 to 10^{11} pfu. The term pfu (plaque forming unit) corresponds to the infectivity of a virus solution, and is determined by infecting an appropriate cell culture and measuring, generally after 15 days, the number of plaques of infected cells. The technique for determining the pfu titre of a viral solution are well documented in the literature.

In a preferred embodiment, the composition comprises an adenovirus comprising the anti-angiogenic factor gene, e.g., ATF gene (AdATF) or angiostatin (AdK3), in a concentration of about 1×10^9 pfu/100 μl.

The compositions according to the invention are particularly useful for administration to tumors.

Tumors. The present invention is directed the treatment of tumors, particularly solid tumors. Examples of solid tumors that can be treated according to the invention include sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

In another embodiment, dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung. Thus, the present invention provides for treatment of conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, *Basic Pathology*, 2 d Ed., W. B. Saunders Co., Philadelphia, pp. 68–79). Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder. For a review of such disorders, see Fishman et al., 1985, *Medicine*, 2 d Ed., J. B. Lippincott Co., Philadelphia.

The present invention is also directed to treatment of non-malignant tumors and other disorders involving inappropriate cell or tissue growth augmented by angiogenesis by administering a therapeutically effective amount of a vector of the invention to the tissue undergoing inappropriate growth. For example, it is contemplated that the invention is useful for the treatment of arteriovenous (AV) malformations, particularly in intracranial sites. The invention may also be used to treat psoriasis, a dermatologic condition that is characterized by inflammation and vascular proliferation; and benign prostatic hypertrophy, a condition associated with inflammation and possibly vascular proliferation. Treatment of other hyperproliferative disorders is also contemplated.

Methods of administration. According to the invention, the preferred route of administration to a tumor is by direct injection into the tumor. The tumor can be imaged using any of the techniques available in the art, such as magnetic resonance imaging or computer-assisted tomography, and the therapeutic composition administered by stereotactic injection, for example.

Alternatively, if a tumor target is characterized by a particular antigen, a vector of the invention can be targeted to the antigen as described above, and administered systemically or subsystemically, as appropriate, e.g., intravenously, intraarterioally, intraperitoneally, intraventricularly, etc.

Combination Therapies. Although the methods of the invention are effective in inhibiting tumor growth and metastasis, the vectors and methods of the present invention are advantageously used with other treatment modalities, including without limitation surgery, radiation, chemotherapy, and other gene therapies.

For example, the vectors of the invention can be administered in combination with nitric oxide inhibitors, which have vasoconstrictive activity and reduce blood flow to the tumor.

In another embodiment, a vector of the invention can be administered with a chemotherapeutic such as, though not limited to, taxol, taxotere and other taxoids [e.g., as disclosed in U.S. Pat. Nos. 4,857,653; 4,814,470; 4,924,011, 5,290,957; 5,292,921; 5,438,072; 5,587,493; European Patent No. 0 253 738; and International Patent Publication Nos. WO91/17976, WO93/00928, WO93/00929, and WO9601815], or other chemotherapeutics, such as cis-platin (and other platin intercalating compounds), etoposide and etoposide phosphate, bleomycin, mitomycin C, CCNU, doxorubicin, daunorubicin, idarubicin, ifosfamide, and the like.

In still another embodiment, a vector of the invention can be administered in conjunction with another gene therapy for tumors, such as but by no means limited to p53 or analogues thereof such as CTS-1 [WO97/04092], thymidine kinase (TK), anti-RAS single chain antibodies, interferon-α or interferon-γ, etc., as described above. Any vector for gene therapy can be used in conjunction with the present invention, such as a viral vector or naked DNA. In a preferred embodiment, a single vector (virus or DNA) is used to deliver genes coding for both an anti-angiogenesis factor and another tumor therapy gene.

In another aspect, the present invention provides for regulated expression of the anti-angiogenic factor gene in concert with expression of proteins useful in the context of treatment for proliferative disorders, such as tumors and cancers, when the heterologous gene encodes a targeting marker or immunomodulatory cytokine that enhances targeting of the tumor cell by host immune system mechanisms. Examples of such heterologous genes for immunomodulatory (or immuno-effector) molecules include, but are not limited to, interferon-α, interferon-γ, interferon-β, interferon-ω, interferon-τ, tumor necrosis factor-α, tumor necrosis factor-β, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM)-1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

The present invention will be better understood be reference to the following Examples, which are provided by way of exemplification and not by way of limitation.

EXAMPLE 1

Gene Therapy With ATF Inhibits Tumor Growth and Metastasis

Example 1 demonstrates that expression of the uPA/uPAR antagonist ATF (amino terminal fragment of urokinase) inhibits tumor growth and metastasis. A defective adenovirus expressing murine ATF from the CMV promoter (AdmATF) was constructed. A single intratumoral injection of AdmATF inhibited growth of pre-established tumors in two different murine models, and delayed tumor dissemination. These effects were correlated with a remarkable inhibition of neovascularization within, and at the immediate vicinity of, the injection site. The magnitude of this effect was particularly remarkable in the ability of murine ATF to inhibit angiogenesis of a human-derived tumor.

Methods

Recombinant adenoviruses. AdmATF is an E1-defective recombinant adenovirus that expresses the murine ATF gene from the CMV promoter. Plasmid pDB1519 16 was used as starting material to introduce a stop codon after residue 135 of mature uPA. Briefly, the uPA-encoding sequences (including its signal peptide) were isolated, restricted by NsiI, and residues 128 to 135 followed by a stop codon were reintroduced as a synthetic fragment. The ATF open reading frame was then inserted between the CMV promoter and the SV40 late polyadenylation signal sequence, generating plasmid pEM8-mATF. This plasmid also carries the first 6.3 kb of the Ad5 genome except that the ATF expression cassette has been inserted between position 382 and 3446, in place of the E1 genes (FIG. 1A). AdmATF was constructed in 293 cells by homologous recombination between pEM8-mATF and ClaI-restricted AdRSVbGal DNA 25. Individual viral plaques were isolated onto 293-derived cell monolayers grown in soft agar, amplified onto fresh 293 cells and viral DNA was extracted 26. EcoRI, EcoRV and AvrII+NdeI restriction analyses confirmed the identity and clonality of the recombinant adenovirus. AdCO1 is a defective control adenovirus that is identical to AdmATF except that it does not carry any transgene expression cassette in place of E1. Both viruses were propagated in 293, a human embryonic kidney cell line that constitutively expresses the E1 genes of Ad5 27. Viral stocks were prepared and titrated as described 25. Unless otherwise stated, MDA-MB-231 cells and Lewis lung carcinoma (LLC) cells were infected at a multiplicity of infection (MOI) of 300 PFU/cell. These infection conditions were previously shown to translate respectively into 80 and 65% of b-galactosidase-expressing cells when virus AdRSVbGal 25 was used.

Northern blot analysis. Subconfluent MDA-MB-231 cell cultures were infected with AdmATF or AdCO1, and total RNA was extracted 24 hr post-infection (p.i.) by the RNAZOL procedure (Biogentex, Inc), and polyadenylated RNAs were purified. The samples were run in a 1% formaldehyde agarose gel, and transferred onto Hybond N membranes (Amersham). The membranes were prehybridized with denatured sonicated salmon sperm DNA (100 μg/ml) for 1 hr at 42° C. in 10 ml of 50% deionized formamide, 0.2% SDS, 5× Denhardt's solution, and incubated overnight with a random-primed (^{32}P)-labeled 1.2 kb XbaI-HindIII fragment from murine uPA cDNA (16). The membranes were washed twice in 2×SSC/0.1% SDS for 1 hr at 50° C., once in 0.1×SSC for 30 min, and expose Kodak-XAR-5 films for 1 hr at room temperature.

Western blot analysis. Supernatants from virally-infected cells were collected 24 hr p.i., run in a 12.5% SDS-polyacrylamide gel (400 μg of protein per lane), prior to transfer onto a nitrocellulose membrane (Schleicher & Schuell). After incubation for 1 hr in blocking buffer, the membranes were incubated for 1 hr with a polyclonal serum raised against murine uPA (Pr. R Lijnen, Leuven, Belgium), then for an additional hour with a horse-radish peroxidase-conjugated goat anti-rabbit serum (Dako). The membranes were washed three times in PBS-Tween buffer, and incubated with 3-Amino-9-ethyl-Carbazole (AEC) for 5 min.

Inhibition of cell-associated proteolysis. Native uPA was first dissociated from its cell surface receptor by submitting LLC cell monolayers to a 3 min acidification in glycine-HCl (pH 3), followed by incubation in 0.5 M HEPES buffer. The cells were then incubated for 20 min at 37° C. with the supernatant of AdCO1- and AdmATF-infected 293 cells. After 3 washes in PBS/0.1% BSA, the cells were incubated at 37° C. for 20 min with 1 nM of murine uPA (Pr. R. Lijnen, Leuven, Belgium). Unbound uPA was then removed by washing in PBS, and cell-associated uPA was quantified by adding 0.4 μM of human plasminogen and plasmin substrate S-2251 (Kabi Vitrum, Sweden).

In vitro invasion assay. Twenty four hr p.i., LLC cells were detached with 1 mM EDTA, washed in PBS, and resuspended in FCS-free MDEM medium supplemented with 0.1% BSA. Invasion assays were carried out in a transwell unit as described (19). Briefly, polycarbonate filters of 1.2 μm pore size (Transwell, Costar) were coated with 160 μg Matrigel (Becton Dickinson) and dried. The lower chambers of the Transwell units were filled with human fibroblast-conditioned medium containing 10 ng/ml EGF, and the upper chambers were seeded with 3×10^5 infected cells. After 24 hr incubation at 37° C., the number of cells that had reached the lower chamber was determined under a light microscope following staining with Giesma.

Syngeneic tumor model. Lewis lung carcinomas were serially passaged onto C57BL/6 syngeneic mice. Briefly, C57BL/6 implanted subcutaneously with a LLC tumor were sacrificed when the tumor had reached a volume of 600–1200 mm^3. Tumor cells were resuspended in a 0.9% saline solution following filtration through a cotton sieve, and 2×10^6 cells (0.5 ml) were subcutaneously implanted to the dorsa of 6–7 weeks-old C57BL/6 female mice. After 5 days, the tumors had reached a size of approximately 20 mm^3, and they were injected with 0.2 ml of PBS (n=8), or 10^9 PFU (0.2 ml) of AdCO1 (n=10) or AdmATF (n=10). The size of the primary tumor was measured at day 5, 10 and 15 p.i. At day 16 p.i., the number of lung metastases was assessed 3 hr after an intraperitoneal injection of 65 mg BrdU. Lung tissues were removed, fixed overnight in acetic formaldehyde acid (AFA), and paraffin sections were incubated 15 min in 4 N HCl, neutralized and saturated by washing twice for 15 min in PBS/0.5% BSA/0.1% Tween 20 prior to incubation with peroxidase-labeled mouse anti-BrdU monoclonal antibody (Boehringer) for 45 mm at 37° C., and AEC. BrdU-positive foci were quantified under a light microscope at a magnification of 25.

Athymic murine model. Cultured MDA-MB-231 cells (ATCC HTB 26) were harvested, washed, resuspended in PBS at 1.5×10^7 cells/ml, and 3×10^6 cells were subcutaneously injected in the dorsa of 6–7 weeks old nude mice. When the tumors had reached a volume of 15–20 mm^3 (i.e., after 11 days), the animals received an intratumoral injection of 10^9 PFU of AdmATF (n=5) or AdCO1 (n=5), or PBS (n=5), and the size of the tumors was monitored until day 52 p.i., after which the animals were sacrificed and the extent of intratumoral vascularization was assessed as described (28). Briefly, tumor tissues were fixed overnight in AFA,

transferred to 100% ethanol, embedded in paraffin and 5 μm thick sections were prepared. After toluene treatment and rehydration, the sections were permeabilized with 2 μg/ml proteinase K at 37° C. for 15 min. Endogenous peroxidase activity was quenched by 0.3% H_2O_2 for 15 min. The sections were washed with PBS, incubated 15 min in 7.5% BSA, and incubated 30 min with a rabbit polyclonal serum raised against human vWF (Dako). After two washes in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG antibodies for 30 min, washed, and incubated with streptavidin-peroxidase for 15 min prior to addition of AEC. Neovascular hotspots were first identified at low magnification and vWF-positive microvessels were quantified. Meyer's hematoxylin was used for counterstaining as described (28).

To evaluate AdmATF infection on tumor establishment, confluent MDA-MB-231 cells were first infected with AdmATF or AdCO1 at an MOI of 50 PFU/cell. The cells were washed 24 hr p.i., resuspended in 120 μl PBS, mixed with 80 μl ice-cold Matrigel, and 1.3×10^6 cells were subcutaneously implanted into the dorsa of nude mice. Tumor establishment and growth were followed until day 51 after implantation.

Results

Molecular and functional characterization of AdmATF. AdmATF is a defective recombinant adenovirus that expresses murine ATF from the CMV promoter whereas AdCO1 is an "empty" control adenovirus (FIG. 1A). In vitro studies were first carried out to characterize AdmATF with regards to its ability to express a functional uPA antagonist following infection. ATF gene expression was demonstrated by northern analysis of poly(A+) RNAs extracted from MDA-MB-231 cells infected for 24 hr with AdmATF, but not AdCO1 (FIG. 1B). Secretion of ATF by AdmATF-infected cells was demonstrated for 293, LLC and MDA-MB-231 cells by Western blot analysis. For example, an ATF-specific polypeptide with a molecular weight corresponding to that of the mature peptide (15.3 kDa) is uniquely detected in the medium from 293 cells infected for 24 hr p.i. with AdmATF (FIG. 1C).

ATF is a potent antagonist of uPA binding to its cell surface receptor (uPAR), and disruption of this complex is known to greatly inhibit the conversion of inactive plasminogen into plasmin. LLC cell-associated plasmin conversion was thus measured to assess the functionality of ATF secreted by AdmATF-infected cells. As a prerequisite, we checked that LLC cells displayed significant levels of cell-associated uPA activity (data not shown), implying that they secrete uPA and express uPAR. Plasmin conversion/activity was significantly reduced when endogenous uPA had been previously removed from the cell surface by a mild acid treatment prior to incubation with the supernatant of AdmATF-infected 293 cells and addition of 1 nM murine uPA (FIG. 2A).

The uPA/uPAR complex is also crucial to cell motility. An in vitro cell invasion assay was used to confirm the functionality of AdmATF. LLCs cells were infected with AdmATF or AdCO1, and the number of cells that had migrated through a matrix-coated membrane was determined after 24 hr (FIG. 2B). Quantification of the data demonstrated that AdmATF infection inhibited LLC invasiveness by 65% (n=5) as compared to AdCO1 control infections.

Intratumoral injection of AdmATF inhibits tumor growth and dissemination. We first used the Lewis lung carcinoma-C57B1/6 syngeneic model to evaluate the antitumoral effects associated with a single intratumoral administration of AdmATF. Five days after subcutaneous implantation, the tumors were injected with 10^9 PFU of AdmATF, 10^9 PFU of AdCO1, or PBS, and tumor growth was monitored until day 15 p.i. As shown in FIG. 3, an overall inhibition was specifically observed in the AdmATF-treated group. The animals were then sacrificed at day 16 p.i., and lung metastases were numbered by counting the number of BrdU-positive foci. Whereas metastases were apparent in all animals injected with PBS (n=8), 7 out of 9, and only 3 out of 9 scored positive within the AdCO1- and AdmATF-treated groups, respectively. The average number of BrdU-positive foci per lung sections was also reduced in the AdmATF-treated group (2.7) as compared to that in the AdCO1-treated (6.3) and PBS-treated (6.6) groups. A single intratumoral administration of AdmATF therefore significantly inhibited tumor growth and lung dissemination in this highly aggressive model. In a separate experiment, tumor-bearing animals were infected with AdCO1 or AdmATF, and the tumors extracted at day 10 and 20 p.i. for macroscopic inspection. While AdCO1-injected LLC tumors displayed an intense vascularization at both time points, tumors from the AdmATF-treated group displayed only marginal vascularization (FIG. 4).

The antitumoral effects of AdmATF are exerted at the level of angiogenesis. To specifically evaluate the sole inhibition of angiogenesis for tumor growth, we studied adenovirus-mediated delivery of the murine uPA/uPAR antagonist in the human-derived MDA-MB-231 breast carcinoma model implanted into athymic mice. A direct action of murine ATF on the tumor cells should be minimal in this model as murine uPA binds human uPAR 200-fold less efficiently than murine uPAR. Eleven days after subcutaneous tumor cell inoculation, the animals received a single intratumoral injection of 10^9 PFU of AdmATF, 109 PFU of AdCO1, or PBS, and tumor growth was monitored until day 52 p.i. While no significant effect were apparent until day 32 p.i., an arrest of tumor growth then became evident in the AdmATF-infected, but not the AdCO1-infected group (FIG. 5). Mice were sacrificed at day 52 p.i., and intratumoral angiogenesis was assessed by visualization of von Willebrand Factor (vWF)-immunoreactive vessels (FIG. 6A). An average of 4 to 6 vessels were detected within the sections from the AdmATF-treated tumors as compared to 18 to 20 in the sections from the AdCO1-injected tumors. Tumors injected with AdmATF also displayed little peripheral neovascularization as compared to their AdCO1-treated counterparts (FIG. 6B). When MDA-MB-231 cells were first infected in vitro before subcutaneous inoculation in the presence of Matrigel, tumors became apparent in the AdCO1-treated group as early as 7 days post-implantation. A tumor of limited size was apparent in only one animal from the AdmATF-treated group (n=5), in sharp contrast to the larger tumors present in 4 out of 5 animals from the AdCO1-infected group. Again, the tumor that had developed following inoculation of AdmATF-infected tumor cells was less vascularized than those that developed following inoculation of AdCO1-infected cells (data not shown).

Discussion

We have studied the antitumoral effects associated with the local delivery of the amino-terminal, non-catalytic, fragment of urokinase (ATF), a potent antagonist of urokinase binding to its receptor (uPAR) at the surface of both tumor (19, 20) and endothelial cells (22, 23). In vivo delivery of ATF was achieved by intratumoral administration of a

defective adenovirus that expresses a secretable ATF molecule of murine origin from the CMV promoter (AdmATF). To exclude non-specific cytotoxic effects consecutive to virus infection (29), an "empty" otherwise isogenic adenovirus (AdCO1) was used as a control virus throughout the study. This is an important control also because recombinant adenoviruses can use the aVb3 integrin for infection (30), a cell surface receptor somehow involved in tumor growth and angiogenesis (31).

A single intratumoral injection of AdmATF is efficient in reducing tumor growth (FIG. 3) and delaying dissemination to the lungs in the aggressive LLC-C57BL/6 syngeneic murine model. Murine ATF apparently partly exerted these effects by inhibiting the invasiveness of the tumor cell themselves (FIG. 2B), a result consistent with the inhibition of cell-associated proteolysis following AdmATF infection (FIG. 2A). ATF-based antagonists are also potent inhibitors of endothelial cells motility (22, 23), suggesting that inhibition of tumor angiogenesis may have also contributed to the effects observed in this model. Indeed, LLC tumors injected with AdmATF displayed very little vascularization as compared to AdCO1-infected control tumors (FIG. 4). That specific AdmATF-mediated tumor growth inhibition became evident at late time p.i. but not so much at early time likely results from lesser requirements of smaller tumors (typically below 300 mm3, see FIG. 3 and FIG. 5) for neovascularization to provide the growth nutrients (for a review see 24).

Inhibition of LLC cells dissemination to the lungs was only transient as the survival rate from the AdmATF-treated group was only slightly extended (less than 30 days after tumor implantation) as compared to that from the AdCO1-treated group (less than 25 days). The effects of AdmATF injection on tumor cells dissemination may be explained either because the tumor cells were frozen following AdmATF infection, and/or because few vessels were available for entry into the vasculature. That dissemination did eventually occur suggests that some tumor cells may have had already reached the vasculature at the time of AdmATF injection. Alternatively, infection with E1-deleted adenoviruses is also typically associated with a rapid clearance of the infected cells in C57BL/6 mice immunotolerant for the transgene product (29), and ATF is a small molecule that exhibits a very short half-life in vivo.

Preclinical data indicate that the uPA/uPAR complex is critically involved in controlling cell migration, including that of endothelial cells. For example, an ATF-IgG fusion protein with an extended in vivo half-life has been shown to inhibit angiogenesis and tumor growth in a bFGF-enriched Matrigel murine model (23). The present study provides evidence that the antitumoral effects of uPA/uPAR antagonists are essentially exerted by controlling intratumoral and peripheral angiogenesis: whereas the antitumoral effects of AdmATF-mediated gene delivery may have been multifactorial as both tumor and endothelial cells are potential targets in the syngeneic tumor model, this is not the case in the MDA-MB-231/athymic murine model because mATF is a poor antagonist of uPA/uPAR complex formation at the surface of human cells, including MDA-MB-231 (32). A remarkable feature that emerged in the MDA-MB-231 model was the efficacy of AdmATF in preventing tumor growth (FIG. 5) and neovascularization within and at the vicinity of the tumor (FIG. 6). In contrast, tumors infected with the control adenovirus were still capable of "attracting" adjacent vessels. The antitumoral properties of AdmATF are further illustrated in this model by the reduced efficacy of tumor establishment following infection.

Malignant tumors are life-threatening because they invade and abrogate the function of vital organs at distant sites, emphasizing the importance of targeting angiogenesis to fight cancer (33; see also 34). First, growth of primary tumors relies on neovascularization to provide the required nutrients. Second, metastases have also been reported to undergo apoptosis in the absence of neovascularization (35). Furthermore, growing capillaries within the tumor are "leaky": they exhibit a fragmented basal membrane (36), a prerequisite for efficient penetration of the tumor cells into the vasculature (37). The overall results of this study demonstrate that significant antitumoral effects can be achieved following a single intratumoral administration of a recombinant adenovirus expressing a potent antagonist of uPA/uPAR function at the cell surface, and that these effects mostly result from an inhibition of angiogenesis. Applying this approach to invasive solid tumors is certainly attractive for cancer gene therapy because of the pleiotropic clinical effects expected following inhibition of tumor angiogenesis.

EXAMPLE 2

Gene Therapy With Angiostatin Inhibits Tumors In Vivo

Example 2 demonstrates that expression of the amino terminal fragment of human plasminogen (angiostatin K3) inhibits tumor growth in vivo by blocking endothelial cell proliferation associated with a mitosis arrest. The antitumoral effects that follow the local delivery of the N-terminal fragment of human plasminogen (angiostatin K3) have been studied in two xenograft murine models. Angiostatin delivery was achieved by a defective adenovirus expressing a secretable angiostatin K3 molecule from the CMV promoter (AdK3). In in vitro studies, AdK3 selectively inhibited endothelial cell proliferation, and disrupted the G2/M transition induced by M-phase-promoting factors. AdK3-infected endothelial cells showed a marked mitosis arrest that correlated with the downregulation of the M-phase phosphoproteins. A single intratumoral injection of AdK3 into pre-established rat C6 glioma or human MDA-MB-231 breast carcinoma grown in athymic mice was followed by a significant arrest of tumor growth, that was associated with a suppression of neovascularization within and at vicinity of the tumors. AdK3 therapy also induced a 10-fold increase in apoptotic tumor cells as compared to control adenovirus. The data support the concept that targeted anti-angiogenesis, using adenovirus-mediated gene transfer, represents a promising strategy for delivering anti-angiogeneic factors as bolus injections of anti-angiogenic proteins still present unsolved pharmacological problems.

Methods

Construction of AdK3. AdK3 is an E1-defective recombinant adenovirus that expresses the N-terminal fragment of human plasminogen (up to residue 333) from the CMV promoter. Human Plg cDNA was obtained from plasmid PG5NM119. A fragment encoding the 18 aa signal peptide of Plg, followed by the first 326 residues of mature Plg was first subcloned between the BamHI and ScaI sites of plasmid pXL2675. A synthetic oligodeoxynucleotide encoding residues 327 to 333 followed by a stop codon was then added, prior to inclusion between the CMV promoter and the SV40 late polyadenylation signal. This expression cassette was then inserted between the EcoRV and BamHII sites of plasmid pCO5 to generate plasmid pCO5-K3. AdK3 was constructed in 293 cells by homologous recombination between pCO5-K3 and ClaI-restricted AdRSVβgal DNA

[25]. Individual plaques were isolated onto 293-derived cell monolayers, amplified onto fresh 293 cells and viral stocks were prepared as described [25]. AdCO1 is a control virus that is identical to AdK3 except that it does not carry any expression cassette.

Cell lines maintenance and infection. C6 glioma cells (ATCC CCL-107) and MDA-MB 231 cells (ATCC HTB 26) were cultured in DMEM with 10% of fetal calf serum (FCS). Viral infection was performed with 5% FCS. Human Microcapillary Endothelial Cells (HMEC-1) [49] were cultured in MCDB 131 supplemented with 20% of FCS, 1 mM L-glutamine, 1 μg/ml of hydrocortisone, 10 ng/ml of epithelium growth factor and infection was performed in the same medium but with 10% of FCS and 3 ng/ml of recombinant human b-FGF (R&D system). The multiplicity of infection (MOI) was chosen as to obtain between 80% to 100% infected cells as judged by X-GAL staining following infection with virus AdRSVβGal.

Western blot analysis. Subconfluent cells were infected with AdK3 or AdCO1 at an MOI of 300 plaque-forming units (PFU)/cell. Cell culture supernatants were collected 48 to 96 hr post-infection (p.i.). For in vivo immunological analysis of the K3 angiostatin molecule, the tumors were collected at day 10 p.i., frozen in liquid nitrogen, powdered, extracted with lysis buffer (10 mM NEM, 1% triton X100, 1 mM PMSF, 0.1 M NH_4OH) and centrifuged at 12000 rpm at 4° C. The samples with 300 μg o protein were run in a 10% SDS-polyacrylamide gel, prior to being transferred onto a nitrocellulose membrane (Schleicher & Schuell). 100 ng human Plg (Stago) was run as a control. After 2 hr incubation in blocking buffer (TBS-5% milk-0.05% Tween 20), the membranes were incubated for 1 hr with anti-human Plg MAb A1D12 [50], 1 hr with a horseradish peroxidase-conjugated goat anti-mouse serum (Biosys). After washing, the membranes were detected with ECL bioluminescence kit (Amersham, UK). To detect the MPM-2 phosphoepitope, the extracts were prepared from the HMEC-1 cell 96 hr p.i. and probed with the specific mitotic MPM-2 MAb (DAKO).

Proliferation assay. Tumor or HMEC-1 cells were infected with AdK3 or AdCO1 at the indicated MOI for 12 hr. The cells were collected with 1 mM EDTA, washed twice with PBS and resuspended. They were seeded into 96-well culture plates (5000 cells/well) and cultured for 72 hr. In addition, HMEC-1 cells were cultured in MCDB131 medium containing 40, 20 or 10% supernatant of AdK3 or AdCO1-transduced C6 glioma cells. Supernatants from virally-infected C6 cells were collected 96 hr p.i., heated 30 min at 56° C. in order to inactivate the virus, concentrated 10 times and dialyzed against PBS. Cells were quantified with a cell proliferation assay kit using a MTS tetrazolium compound (Promega).

Formation of capillary tube in a fibrin matrix model. This model was devised according to the method of Pepper et al [51] using Calf Pulmonary Artery Endothelial cells (CPAE) (ATCC CCL 209) infected for 12 hr with AdK3 or AdCO1 at an MOI of 600.

Whole blood lysis assay. Whole blood clot lysis was performed by mixing 80 U/ml of tissue-plasminogen activator, 250 μl of culture supernatant obtained 4 days p.i. with AdK3 or AdCO1, and 500 μl of citrate-anti-coagulated whole blood collected from healthy donors. Coagulation was triggered by adding 1 U/ml of thrombin and of 12 mM Ca++. The extent of clot lysis was determined by lysis time and by following the kinetics of soluble D-Dimers as described [52].

Immunoflow cytometry. HMEC-1 were infected for 96 hr with AdK3 or AdCO1 at an MOI of 300 PFU/cell. The cells were collected, permeabilized with triton X100, incubated with iodide propidium (20 μg/ml) and ribonuclease A (100 μg/ml) for 30 min at room temperature to label DNA, prior to incubation with mitotic MPM-2 antibody as described [53]. FITC-conjugated anti-mouse IgG antibodies were used to detect MPM-2 phosphoepitope. The experiment was performed in a Coulter EPICS Profile II flow cytometer and the data were analysed by Multicycle software (Phoenix Flow Systems, San Diego, Calif.).

Athymic murine models. Cultured C6 glioma cells and MDA-MB-231 cells were harvested, washed, resuspended in PBS at 1.5×10^7 and 0.25×10^7cells/ml respectively and a volume of 200 μl subcutaneously injected into the dorsa of 6–7 weeks old nude mice. When the tumors had reached a volume of 20 mm^3, the animals received an intratumoral injection of 10^9 PFU of either AdK3 (n=6), or AdCO1 (n=6), or PBS (n=6). Tumor size was monitored until day 10 p.i. for the C6 glioma model, and day 42 p.i. for the MDA-MB-231 model.

To assess the effect of AdK3 infection on tumor establishment and progression, MDA-MB-231 and C6 cells were infected for 24 hr at an MOI of 50 and 100 PFU/cell, respectively, prior to subcutaneous inoculation into the dorsa of nude mice (n=6). Infected MDA-MB-231 cells are less tumorigenic than infected C6 cells so 80 μl ice-cold Matrigel (Becton Dickinson) had to be added to 120 μl of PBS prior to subcutaneous implantation (10^6 MDA-MB-231 or 0.25×10^6 C6 cells). Tumor establishment and growth were followed until day 25 (MDA-MB-213) or day 22 (C6) p.i. A Student's t-test was used for statistical analysis.

Immunohistochemistry. Tumor tissues were fixed in alcohol formalin acetic acid, embedded in paraffin and 5 μm sections were prepared. After toluene treatment and rehydration, the sections were pretreated three times for 5 min in a microwave oven in 10 mM citrate buffer (pH 6.0), quenched by 3% H_2O_2 for 5 min to remove endogenous peroxidase activity, washed in PBS, then incubated with a rabbit polyclonal serum raised against human von Willebrand factor (vWF; Dako, dilution 1:200) for 60 min. After 3 washes, the sections were incubated with biotinylated goat anti-rabbit IgG antibodies for 30 min., washed, and incubated with streptavidin-peroxidase for 30 min. prior to addition of 3-Amino-9-ethyl-carbazole. Meyer's hematoxylin was used for counterstaining. Apoptotic cells within the section were detected by a kit using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method (TUNEL) (Boehringer Mannheim). For proliferating cell nuclear antigen (PCNA) staining procedure included a biotinylated mouse anti-PCNA antibody (Pharmingen, dilution 1:100) followed by streptavidin peroxidase and substrate revelation.

Results

Molecular characterization of AdK3. Recombinant AdK3 carries a CMV-driven N-terminal fragment of human Plg that includes the first three kringle domains of the angiostatin molecule [47], whereas AdCO1 is an "isogenic" control adenovirus that does not encode any expression cassette (FIG. 7A). Secretion of the K3 molecule in the culture media 2–3 days after infection with AdK3 was demonstrated for HMEC-1, C6 and MDA-MB-231 cells by Mab A1D12 immunoblotting, whereas no signal was detected following infection with AdCO1 (FIG. 7B). The secreted immunoreactive peptide appeared as a doublet with a molecular weight of 36 and 38 kDa, most likely reflecting a different extent of N-glycosylation at Asn^{289} as described for Plg [54, 55].

Functional characterization of AdK3. Transduction of HMEC-1 by AdK3 resulted in an inhibition of bFGF-stimulated proliferation in a dose-dependent manner at day 3 p.i.: 30% at an MOI of 50, 74% at an MOI of 150, and 97% at an MOI of 300, in sharp contrast to the cells infected with AdCO1 ($P<0.005$). AdK3 did not affect MDA-MB-231 and C6 cell proliferation (FIG. 8A). To assess the paracrine potential of the K3 molecule to exert these effects, virus-free culture media from virally-infected C6 glioma cells were added to HMEC-1 cells. As illustrated in FIG. 8A, we did observe a dose-dependent inhibition of HMEC-1 cell proliferation by C6 cell-secreted angiostatin ($p<0.001$). The addition of AdK3 also significantly inhibited the capillary formation of CPAE cells in fibrin gel with a 55% mean reduction (not shown). Moreover, whole blood clot lysis induced by tPA was not inhibited by the addition of cell culture supernatants from AdK3-infected C6 cells, and the generation of D-Dimers was basically unchanged during the first three hours (1200 ng/ml versus 1150 ng/ml).

AdK3 inhibits mitosis of endothelial cells. To determine if angiostatin is able to block the mitosis of HMEC-1, a flow immunocytometry analysis was performed with the cells labeled with MAb MPM-2 that binds to the phosphorylated proteins specifically present during the M-phase, together with concurrent DNA staining. The results showed that mitosis of AdK3-infected HMEC-1 cells was decreased by 82% relative to AdCO1 infection: only 5% of HMEC-1 cells within the G2/M pic scored positive for MPM-2 following infection with AdK3 as compared to 27% following AdCO1 control infection (FIG. 8C). Western blot analysis was performed from HMEC-1 extracts in order to detect MPM-2 positive proteins as at least 16 mitotic phosphoproteins were usually revealed by MPM-2 with an apparent molecular weight ranging from 40 to more than 200 kDa. As compared to control extracts from non-infected or AdCO1-infected HMEC-1 cells, extracts from AdK3-infected cells exhibited a markedly reduced level of MPM2-reactive phosphoproteins (FIG. 8B).

AdK3 inhibits tumor growth. To induce local secretion of angiostatin, a single dose of 10^9 PFU of AdK3 was injected into 20 mm^3 pre-established human MDA-MB-231 breast carcinoma and rat C6 glioma tumors grown in athymic mice, and tumor growth was monitored. As shown in FIG. 9A, C6 tumors from the AdK3-injected group were significantly smaller than those from the AdCO1 or the PBS control groups: at day 10 p.i., AdK3-injected tumors had reached a mean volume of 278±14 mm^3 versus 1403±142 mm^3 or 1583±259 mm^3 for AdCO1- and PBS-injected tumors, respectively ($p<0.05$). This 80% inhibition correlated with the detection of angiostatin-immunoreactive material (FIG. 7C). As shown in FIG. 9B, tumor growth was similarly inhibited (85%) in the MDA-MB-231 tumor model at day 42 p.i.: 80±4 mm^3 for AdK3-treated tumors versus 563±137 mm^3 for AdCO1- and 530±69 mm^3 for PBS-injected tumors respectively ($p<0.05$).

AdK3 inhibits angiogenesis and induces tumor cell apoptosis in vivo. C6 tumors infected with AdCO1 appeared much more vascularized than their AdK3-infected counterparts (FIG. 10, panels A–B). Intratumoral angiogenesis was thus assessed by vWF-immunostaining of tumor sections as described [28]. vWF-positive hotspots were first localized at low magnification, and vWF-positive vessels were then counted at 200×magnification (FIG. 10, panels E–F). The results indicated a marked reduction of intratumoral vascularization within AdK3-injected tumors (5±2 vWF-positive vessels per field) as compared to the AdCO1-injected control (14±4; $n=5$, $p<0.005$). Tumors in the PBS-injected group exhibited an identical number of vessels (14±3) indicating that the infection conditions used did not interfere with tumor angiogenesis. At the macroscopic level, C6 tumors injected with AdK3 displayed little peripheral neovascularization as compared to their AdCO1-treated counterparts (FIG. 10, panels C–D). Similar results were obtained within MDA-MB-231 tumor sections (4.8±1.2 vWF-immunoreactive vessels/field for AdK3 versus 15.6±3 for AdCO1, $p=0.02$).

Tumor cell apoptosis was then quantified in situ with the C6 tumor samples by the TUNEL method (see Methods). The results indicated a marked increase of apoptotic cells in the AdK3-injected C6 tumors 10 days p.i. (20±9 versus 1–2 apoptotic cells per field for control tumors, $p<0.001$) (FIG. 10, panels G–H). In contrast, the tumor cell proliferation rate was not different among the three animal groups as assessed by PCNA immunostaining (not shown). Ad-angiostatin therapy induced a 10 fold increase in apoptotic tumor cells without affecting the proliferation of these cells, similar to the reported results obtained by daily injections of purified angiostatin.

AdK3 inhibits tumorigenesis. To determine whether inhibition of tumor angiogenesis attenuated tumorigenesis, MDA-MB-231 and C6 cells were first infected for 24 hr prior to injection into the dorsa of nude mice. After 5 days, all the mice from the AdCO1-infected group developed hypervascularized C6 tumors with an average size of 27.4±3.41 mm^3, whereas 20% of animals from the AdK3-infected group remained tumor free after 12 days (FIG. 11). The remaining animals exhibited very small tumors (average size of 0.42±0.05 mm^3) that were hardly vascularized. After 22 days, the tumors that were observed within the AdK3 group were at least 5-fold smaller than those from the AdCO1 group ($n=5$, $p<0.005$; FIG. 11). Similar observations were made with the MDA-MB-231 tumor model (not shown).

Discussion

Angiostatin has been shown to be a physiopathological inhibitor of angiogenesis secreted by primary tumors, driving the metastasis into a dormant state. It was therefore tempting to assess the therapeutic potential of angiostatin on primary tumors. However, systemic and intraperitoneal bolus injections of human angiostatin have underlined difficult pharmacological problems because angiostatin is rapidly cleared from the circulation [46]. A prolonged exposure of purified angiostatin at high doses was indeed required to maintain cytostatic intratumoral concentrations of angiostatin [46]. It was not clear that direct transduction of the tumor and the surrounding tissue with a recombinant virus encoding an angiostatin cDNA would represent a more efficient method of achieving constant intratumoral concentrations of angiostatin. Adenoviruses are appropriate vectors in such a strategy as they can efficiently express their transgene at therapeutic levels in both proliferating and non-proliferating cells (for a review see [37]), allowing to target a wide area for angiostatin production. Thus, a defective adenovirus that expresses the N-terminal fragment (aa 1-333) from human Plg, including its pre-activation peptide and kringles 1 to 3 (AdK3) was constructed.

The use of Mab A1D12, which is specific to human Plg [50] first demonstrated an efficient secretion of angiostatin in the culture media of cells infected with AdK3. The inclusion of the N-terminal pre-activation peptide in the angiostatin molecule did not affect its anti-angiogenic activity since AdK3- but not AdCO1-infected endothelial cells showed a

marked, dose-dependent, arrest in proliferation in vitro (FIG. 8A). Furthermore, the proliferation of MDA-MB-231 or C6 tumor cells was not affected by AdK3-infection demonstrating the restricted action of angiostatin for endothelial cells. Virus-free supernatants from AdK3-infected tumor cell culture also inhibited endothelial cell proliferation, illustrating the paracrine effect of angiostatin secreted by transduced-tumor cells.

Because the kringle domains are important for Plg binding to fibrin and fibrin degradation, it was essential to analyze the effect of this therapy in thrombolysis, a physiological protection against thrombosis in vivo. The angiostatin secreted in the culture medium failed to inhibit tPA-induced whole blood clot lysis in vitro. Although this experiment has not excluded the deleterious competition between angiostatin and Plg to bind to fibrin during thrombolysis in vivo, it indicates that an angiostatic effect could be achieved at a concentration far below that required for abrogating plasminogen-dependent thrombolysis in vivo. This may also suggest that endothelial cells exhibit a receptor that recognizes angiostatin and not intact Plg.

Flow cytometry analysis of endothelial cells infected with AdK3 demonstrated a complete disappearance of the mitotic population positive for MPM-2 MAb [56]. Immunoblot analysis revealed that M-phase phosphoproteins reactive to MPM-2 MAb were indeed downregulated in angiostatin-treated endothelial cells, in sharp contrast with control endothelial cells. This observation should be helpful to define the mechanism by which angiostatin abrogates the proliferation of endothelial cells. We also showed that angiostatin disrupted the G2/M transition induced by M-phase-promoting factor (MPF), composed of cdc2 and its associated regulatory subunit, cyclin B [57]. MPF phosphorylated proteins, reactive with MPM-2 MAb, are involved in major alterations of cellular structures and activities for an efficient progression to mitosis. The reason why active MPF was lacking in AdK3-transduced endothelial cells must be further investigated.

A single intratumoral injection of AdK3, but not of AdCO1 was shown to dramatically inhibit primary tumor growth in two pre-established xenograft murine models. This inhibitory effect on tumor growth was tightly correlated with a markedly decreased vascularization within, and at the vicinity of the tumors (FIG. 10), together with the detection of angiostatin-immunoreactive material in the tumor extracts (FIG. 7C). C6 glioma is a highly vascularized tumor due to its VEGF overexpression [58]. Interestingly, the AdK3-transduced C6 glioma apparently failed to establish a vascular network within the tumor mass to support rapid and extensive growth (FIG. 10), and this failure translated to more than 80% inhibition of tumor growth. vWF immunostaining of tumor sections also revealed a significant reduction of neoangiogenesis in the AdK3-treated tumors: well formed vessels with a mature lumen were frequently observed in control C6 tumors, but not in AdK3-treated C6 glioma (FIG. 10). This decrease in vessel density was associated with a 10-fold increase in tumor cells apoptosis and no apparent modification of the tumor cell proliferation index, probably because (i) of the lack of endothelial-derived paracrine factors, (ii) a reduction in nutrient support, and (iii) hypoxia triggered p53-dependent apoptosis of the tumor cells [59, 60]. In the MDA-MB-231 breast carcinoma model, a single intratumoral injection of AdK3 similarly induced a remarkable inhibition of tumor angiogenesis and growth.

In the course of this study, AdK3-transduced C6 and MDA-MB-231 cells exhibited a lower tumorigenic potential as reflected by a prolonged delay for AdK3-infected cells to develop into visible tumors following implantation.

Angiostatic therapy using recombinant adenoviruses has been shown to be experimentally plausible and efficient. The possibility of delivering more than one angiostatic factor could also synergize to arrest tumor growth. It is also envisioned that its association with cytotoxic approaches may be particularly potent to improve the clinical outcome of malignant diseases.

REFERENCES

The following reference citations, which are cited by number in the Background and Examples of the invention, are specifically incorporated herein by reference in their entireties:

1) E. Bacharach, A. Itin and E. Keshet. In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proc. Natl. Acad. Sci. USA 89, 10686–10690 (1992).

2) P. R. M. Mignatti and D. B. Rifkin. Biology and biochemistry of proteinases in tumor invasion. Physiol. Rev. 73, 161–195. (1993).

3) S. Imren, D. B. Kohn, H. Shimada, L. Blavier and X. DeClerck. Overexpression of tissue inhibitor of metalloproteinases-2 by retroviral-mediated gene transfer in vivo inhibits tumor growth and invasion. Cancer Res. 56, 2891–2895 (1996).

4) M. Ploug, E. Ronne, N. Behrendt, A. L. Jensen and F. Blasi. Cellular receptor for urokinase plasminogen activator: carboxyl-terminal processing and membrane anchoring by glycosylphosphatidylinositol. J. Biol. Chem. 266, 1926–1933 (1991).

5) A. L. Roldan, M. V. Cubellis, M. T. Masucci, N. Behrendt, L. R. Lund, K. Dano, E. Appella and F. Blasi. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. EMBO J. 9, 467–474 (1990).

6) V. Ellis, N. Behrendt and K. Dano. Plasminogen activation by receptor-bound urokinase, a kinetic study with cell-associated and isolated receptor. J. Biol. Chem. 266, 12752–12758 (1991).

7) C. He, S. M. Wilhelm, A. P. Pentland, B. L. Marmer, G. A. Grant, A. Z. Eisen and G. I. Goldberg. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc. Natl. Acad. Sci. USA 86, 2632–2636 (1989).

8) A. Estreicher, J. Muhlhauser, J. L. Carpentier, L. Orci and J. D. Vassalli. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J. Cell Biol. 111, 783–792 (1990).

9) Y. Wei, D. A. Waltz, N. Rao, R. J. Drummond, S. Rosenberg and H. A. Chapman. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J. Biol. Chem. 269, 32380–32388 (1994).

10) Y. Wei, M. Lukashev, D. I. Simon, S. C. Bodary, S. Rosenberg, M. V. Doyle and H. A. Chapman. Regulation of integrin function by the urokinase receptor. Science 273, 1551–1555 (1996).

11) L. Naldini, L. Tamagnone, E. Vigna, M. Sachs, G. Hartmann, W. Birchmeier, Y. Daikuhara, H. F. B. Tsubouchi and P. M. Comoglio. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J. 11, 4825–4833 (1992).

12) D. Rifkin, D. Moscatelli, J. Bizik, N. Quarto, F. Blei, P. Dennis, R. Flaumenhaft and P. Mignatti. Growth factor

control of extracellular proteolysis. Cell Differ. Dev. 32, 313–318 (1990).

13) M. Schmitt, F. Janicke and H. Graeff. Tumor-associated proteases. Fibrinolysis 6, 3–26 (1992).

14) C. Pyke, P. Kristensen, E. Ralfkiaer, J. Grondhal-Hansen, J. Eriksen, F. Blasi and K. Dano. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am. J. Pathol. 138, 1059–1067 (1991).

15) R. L. Shapiro, J. G. Duquette, D. F. Roses, I. Nunes, M. N. Harris, H. Kamino, E. L. Wilson and D. B. Rifkin. Induction of primary cutaneous melanocytic neoplasms in urokinase-type plasminogen activator (uPA) deficient and wild-type mice: cellular blue Nevi invade but do not progress to malignant melanoma in uPA-deficient animals. Cancer Res. 56, 3597–3604 (1996).

16) D. Belin, J. D. Vassalli, C. Combepine, F. Godeau, Y. Nagamine, E. Reich, H. P. Kocher and R. M. Duvoisin. Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator. Eur. J. Biochem. 148, 225–232 (1985).

17) E. Appella, E. A. Robinson, S. J. Ullrich, M. P. Stoppelli, A. Corti, G. Cassani and F. Blasi. The receptor binding sequence of urokinase: a biological function for the growth factor module of proteases. J. Biol. Chem. 262, 4437–4440 (1987).

18) V. Magdolen, P. Rettenberger, M. Koppitz, L. Goretski, H. Kessler, U. H. Weidle, B. Konig, H. Graeff, M. Schmitt and O. Wilhelm. Systemic mutational analysis of the receptor-binding region of the human urokinase-type plasminogen activator. Eur. J. Biochem. 237, 743–751 (1996).

19) H. Lu, P. Yeh, J. D. Guitton, C. Mabilat, F. Desanlis, 1. Maury, Y. Legrand, J. Soria and C. Soria. Blockage of the urokinase receptor on the cell surface: construction and characterization of a hybrid protein consisting of the N-terminal fragment of human urokinase and human albumin. FEBS Lett. 356, 56–59 (1994).

20) C. W. Crowley, R. L. Cohen, B. K. Lucas, G. Liu, M. A. Shuman and A. D. Levinson. Prevention of metastasis by inhibition of the urokinase receptor. Proc. Natl. Acad. Sci. U.S.A. 90, 5021–5025 (1993).

21) H. Kobayashi, J. Gotoh, M. Fujie, H. Shinohara, N. Moniwa and T. Terao. Inhibition of metastasis of Lewis lung carcinoma by a synthetic peptide within the growth factor-like domain of urokinase in an experimental and spontaneous metastasis modellnt. J. Cancer 57, 727–733 (1994).

22) H. Lu, C. Mabilat, P. Yeh, J. D. Guitton, H. Li, M. Pouchelet, D. Shoevaert, Y. Legrand, J. Soria and C. Soria. Blockage of urokinase receptor reduces in vitro the motility and deformability of endothelial cells. FEBS Lett. 380, 21–24 (1996).

23) H. Y. Min, L. V. Doyle, C. R. Vitt, C. L. Zandonella, J. R. Stratton-Tomas, M. A. Shuman and S. Rosenberg. Urokinase receptor antagonists inhibit angiogenesis and primary tumor growth in syngenic mice. Cancer Res. 56, 2428–2433 (1996).

24) J. Folkman. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine 1, 27–31. (1995).

25) L. D. Stratford-Perricaudet, I. Makeh, M. Perricaudet and P. Briand. Widespread long-term gene transfer to mouse skeletal muscles and heart via an adenovirus vector. J. Clin. Invest. 90, 626–630 (1992).

26) B. Hirt. Selective extraction of polyoma DNA from infected mouse cells cultures. J. Mol. Biol. 26, 365–369 (1967).

27) F. L. Graham, J. Smiley, W. C. Russel and R. Nairn. Characteristics of a human cell line transformed by DNA from human adenovirus type 5J. Gen. Virol. 36, 59–72 (1977).

28) N. Weidner, J. P. Semple and W. R. Welch. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med. 324, 1–8 (1991).

29) J.-F. Dedieu, E. Vigne, C. Torrent, C. Jullien, 1. Mahfouz, J.-M. Caillaud, N. Aubailly, C. Orsini, J. M. Guillaume, P. Opolon, P. Delaère, M. Perricaudet and P. Yeh. Long-term gene delivery into the liver of immunocompetent mice with E1/E4-defective adenoviruses. J. Virol. (1997).

30) T. J. Wickham, P. Mathias, D. A. Cheresh and G. R. Nemerow. Integrins avb3 and avb5 promote internalization but not virus attachment. Cell 73, 309–319 (1993).

31) P. C. Brooks, A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier and D. A. Cheresh. Integrin aVb3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79, 1157–1164 (1994).

32) M. O'Reilly, L. Holmgren, Y. Shing, C. Chen, R. Rosenthal, M. Moses, W. Lane, Y. Cao, E. Sage and J. Folkman. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79, 315–328 (1994).

33) T. P. D. Fan, R. Jaggar and R. Bicknell. Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. TIPS 16, 57–66 (1995).

34) L. Holmgren, M. S. O'Reilly and J. Folkman. Dormancy of micrometastasis: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Med. 1, 149–153 (1995).

35) H. F. Dvorak, J. A. Nagy, J. T. Dvorak and A. M. Dvorak. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am. J. Pathol. 133, 95–109 (1988).

36) L. Liotta, J. Kleinerman and G. Saidel. Quantitative relationships of intravascular tumor cells, tumor vessels and pulmonary metastases following tumor implantation. Cancer Res. 34, 997–1004 (1974).

37) P. Yeh and M. Perricaudet. Advances in adenoviral vectors: from genetic engineering to their biology. FASEB J. 11:615–623 (1997).

38) J. Folkman. What is the evidence that tumors are angiogenesis dependent. *J. Natl. Cancer Inst.* 82, 4–6 (1990).

39) D. Hanahan & J. Folkman. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364 (1996).

40) A. Hori. Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Res.* 51, 6180–6184 (1991).

41) K. J. Kim. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362, 841–844 (1993).

42) M. O. O'Reilly, T. Boehm, Y. Shing, N. Fukai, G. Vasios, W. S. Lane, E. Flynn, J. R. Birkhead, B. R., Olsen, & J. Folkman. Endostatin: An Endogenous Inhibitor of Angiogenesis and tumor Growth. *Cell* 88, 277–285 (1997).

43) C. Clapp, J. A. Martial, R. C. Guzman, F. Rentier-Deirue, & R. I. Weiner. The 16 kDa N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinol.* 133, 1292–1299 (1993).

44) S. K. Gupta, T. Hassel, & J. P. Singh. A potent inhibitor of endothelial cell proliferation is generated by proteolytic

cleavage of the chemokine platelet factor 4. *Proc. Natl. Acad. Sci. USA.* 92, 7799–7803 (1995).

45) Z. Dong, R. Kumar, X. Yang, & I. Fidler. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis Lung Carcinoma. *Cell* 88, 801–810 (1997).

46) M. O'Reilly, L. Holmgren, C. Chen, & J. Folkman. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nature Med.* 2, 689–692 (1996).

47) Y. Cao, R. W. Ji, D. Davidson, J. Schaller, D. Marti, S. Söhndel, S. G. McCance, M. S. O'Reilly, M. Llinas, & J. Folkman. Kringle domains of human angiostatin. *J. Biol. Chem.* 271, 29461–29467 (1996).

48) J. A. Roth & R. J. Christiano. Gene therapy for cancer: what have we done and where are we going. *J. Natl. Cancer Inst.* 88, 21–39 (1997).

49) V. Trochon, C. Mabilat, P. Bertrand, Y. Legrand, F. Smadja-Joffe, C. Soria, B. Delpech & H. Lu. Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro. *Int. J. Cancer* 66, 664–668 (1996).

50) M. Mirshahi, J. Soria, H. R. Lijnen, V. Fleury, O. Bertrand, J. Y. Drouet, J. Caen & C. Soria. A monoclonal antibody directed against an epitope in the NH2-terminal region of native human plasminogen induces a modification of its functional properties. *fibrinolysis and Coagulation, in press* (1997).

51) M. S. Pepper, R. Montesano, J. D. Vassalli & L. Orli. Chondrocytes inhibits endothelial sprout formation in vitro: evidence for involvement of a transforming growth factor β. *J. cell. Physiol.* 146, 170–179(1991).

52) M. Mirshahi, J. C. S. Soria, R. Faivre, H. Lu, M. Courtney, C. Roitsch, D. Tripier & J. P. Caen. Evaluation of the inhibition by heparin and hirudin of coagulation activation by r-tPA induced thrombolysis. *Blood* 74, 1025–1030 (1989).

53) A. Skladanowski & A. K. Larsen. Expression of Wild-type p53 etoposide cytotoxicity in M1 myeloid leukemia cells by facilitated G2 to M transition: Implications for gene therapy. *Cancer Res.* 57, 818–823 (1997).

54) B. K. Lee Sim, M. S. O'Reilly, H. Liang, A. H. Fortier, W. He, J. W. Madsen, R. Lapcevich & C. A. Nacy. A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. *Cancer Res.* 57, 1329–1334 (1997).

55) M. L. Hayes & F. J. Castellino. Carbohydrate of the human plasminogen variant II. Structure of the asparagine-linked oligosaccharide unit. *J. Biol. Chem.* 254, 8772–8776 (1979).

56) F. M. Davis, T. Y. Tsao, S. K. Fowler & P. N. Rao. Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci.* 80, 2926–2930 (1983).

57) R. W. King, P. K. Jackson & M. W. Kirschner. Mitosis in transition. *Cell* 18, 563–571 (1994).

58) K. H. Plate, G. Breier, H. A. Weich & W. Risau. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature* 359, 845–848 (1992).

59) J. Hamada, P. G. Cavanaugh, O. Lotan & G. L. Nicolson. Separate growth and migration factors for large-cell lymphoma cells secreted by microvascular endothelial cells derived from target organs for metastasis. *Br. J. Cancer* 66, 349–354 (1992).

60) G. G. Graeber. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. *Nature* 379, 88–91 (1996).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

What is claimed is:

1. A method for inhibiting the growth or metastasis or both of a tumor comprising introducing a vector into the tumor, the vector comprising a nucleic acid sequence encoding an amino terminal fragment of urokinase that comprises an EGF-like domain, with the exception that the nucleic acid sequence does not encode full length urokinase, wherein the nucleic acid sequence is operably associated with an expression control sequence that provides for expression in a cell of the tumor.

2. The method of claim **1** wherein the expression control sequence comprises a CMV promoter.

3. A method for inhibiting the growth of a tumor comprising introducing into the tumor a defective adenovirus vector comprising a DNA sequence encoding an anti-angiogenic factor operably associated with an expression control sequence that provides for expression of the anti-angiogenic factor in a cell of the tumor, wherein the anti-angiogenic factor comprises an amino terminal fragment of urokinase that comprises an EGF-like domain, with the exception that the anti-angiogenic factor is not full length urokinase.

4. The method of claim **3**, wherein the expression control sequence comprises a CMV promoter.

5. The method of claim **3**, wherein the adenovirus vector contains a deletion in the E1 region.

6. The method of claim **5**, wherein the expression control sequence comprises a CMV promoter.

7. A defective adenovirus vector comprising a gene encoding an anti-angiogenic factor operably associated with an expression control sequence, wherein the anti-angiogenic factor comprises an amino terminal fragment of urokinase comprising an EGF-like domain, with the exception that the anti-angiogenic factor is not full length urokinase.

8. The vector of claim **7**, wherein the expression control sequence comprises a CMV promoter.

9. The vector of claim **7**, wherein the adenovirus vector contains a deletion in the E1 region.

10. The vector of claim **9**, wherein the expression control sequence comprises a CMV promoter.

11. A pharmaceutical composition comprising the defective adenovirus vector according to claim **7** and a pharmaceutically acceptable carrier.

12. A pharmaceutical composition comprising the defective adenovirus vector according to claim **8** and a pharmaceutically acceptable carrier.

13. A pharmaceutical composition comprising the defective adenovirus vector according to claim **9** and a pharmaceutically acceptable carrier.

14. A pharmaceutical composition comprising the defective adenovirus vector according to claim **10** and a pharmaceutically acceptable carrier.

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Amino-Terminal Fragment of Urokinase-Type Plasminogen Activator Inhibits HIV-1 Replication

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CD8⁺ T lymphocytes have been shown to produce unidentified soluble factors active in suppressing HIV-1 replication. In this study, we purified an HIV-1 suppressing activity from the culture supernatant of an immortalized CD8⁺ T cell clone, derived from an HIV-1 infected long-term nonprogressor, and identified this activity as the amino-terminal fragment (ATF) of urokinase-type plasminogen activator (uPA). ATF is catalytically inactive, but suppresses the release of viral particles from the HIV-1 infected cell lines via binding to its receptor CD87. In contrast, cell proliferation and the secretion of an HIV-1 LTR driven reporter gene product were not affected by ATF. These findings suggest that ATF may inhibit the assembly and budding of HIV-1, which provides a novel therapeutic strategy for AIDS. © 2001 Academic Press

Key Words: **HIV; AIDS; CD8; uPA; ATF; uPAR; lipid raft; assembly; budding; GPI-anchored protein.**

CD8⁺ T lymphocytes constitute an important component of the immune response in viral infections. In addition to the MHC class-I-restricted CTL activity, CD8⁺ T lymphocytes have the activity that suppresses HIV-1 replication in the absence of cell killing and the inhibitory effect could be mediated by the soluble factors (1–3). Some soluble factors have been reported to have the HIV-1 suppressive activities with various mechanisms. CC-chemokines, RANTES, MIP-1α, and MIP-1β, were shown to be the major components of CD8⁺ T cell derived HIV suppressive activity (4). They inhibit macrophage-tropic HIV-1 infection by interfering viral entry (5). IL-16 has been also reported a components of CD8⁺ T cell derived HIV suppressive activity (6). IL-16 inhibits HIV-1 mRNA transcription (7, 8). CC-chemokine, MDC, was also shown had anti-viral activity against both T cell-tropic and macrophage-tropic HIV-1 (9). However, they could not account for all of the suppressive effects mediated by CD8⁺ T cell supernatants (3). Therefore, the existence of unidentified HIV-1 suppressor factors has been expected (10–16).

MATERIALS AND METHODS

Preparation of CD8⁺ T cell supernatants. CD8⁺ T cells were isolated from peripheral blood of an HIV-1 positive long-term nonprogressor by positive selection with anti-CD8 antibody coated magnetic beads, and immortalized by coculturing with an HTLV-1 producing cell line MT-2 which had been irradiated with 10,000 rad (4). After a month of cocultivation, cells were cloned by limiting dilution. Clones were maintained in RPMI1640 medium containing 10% fetal calf serum (FCS), 10 units/ml IL-2, and 15% conditioned medium from human peripheral blood mononuclear cells. Clone No. 62 was cultured with PM1000 medium (Eiken Chemical, Tokyo, Japan) supplemented with 5% FCS and 10 units/ml IL-2. Half the volume of supernatant was collected every 3–4 days, then cells were refed with the same vol of fresh medium, for three months of continuous culturing. The collected supernatant was filtered and stored at −80°C.

Purification of the HIV-1 suppressive activity. Acid treatment was performed at pH 2.5 for 24 h at 4°C. The eight steps of column chromatography, included SP sepharose, Blue sepharose, Butyl sepharose, Phenyl sepharose, hydroxyapatite, Sephacryl S-100 HR, Resource S, and a second round of hydroxyapatite, were performed using common chromatographic techniques. The column for the second round of hydroxyapatite chromatography (0.5 × 5 cm, CHT-2, 20 μm; BioRad, Hercules, CA) was equilibrated with 10 mM potassium phosphate, pH 6.35, 0.1% Chaps. Elution was carried out using a 25 ml linear gradient of potassium phosphate (10–400 mM). Fractions of 1 ml each were collected. Samples were assayed for HIV-1 suppressor activity at a final concentration of 0.5%. Peptide mapping and internal sequencing analysis were performed as described (17). The protein concentration of purified ATF was estimated by Sypro-Ruby (Molecular Probes, Inc., Eugene, OR) staining.

Purification of ATF from clinical grade uPA. 50,000 units of crude clinical grade uPA (JCR Pharmaceuticals, Kobe, Japan) was

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applied to a Sephacryl S-100 HR column (1.6 × 60 cm) equilibrated with 10 mM sodium phosphate, pH 6.4, 100 mM NaCl, 0.1% Chaps. Elution was performed with 144 ml of the same buffer. Fractions of 2.5 ml each were collected. Samples were assayed for HIV-1 suppressor activity at a final concentration of 1%.

Cell lines. The chronically infected cell line, TALL-1_{NL4-3}, was constructed by transfection of the TALL-1 $CD4^+$ T cell line with pNL4-3, a plasmid encoding infectious HIV-1 DNA (18) and cloned by limiting dilution. TALL-1_{NL4-3} cells were maintained in the presence of 10 μM AZT since it is susceptible to superinfection. U937 promonocytic cell line was obtained from American Type culture collection (Rockville, MD). The U1 cell line, which is chronically infected with HIV-1, and HUT78 cell line were obtained from the AIDS Research and Reference Program (contributed by T. M. Folks and A. Gazder through R. Gallo, respectively).

Coculture assay. 2×10^4 uninfected cells and 5×10^3 chronically infected cells were seeded in a 48 well plate and inoculated with 600 μl of PM1000 medium containing 5% FCS, 1 ng/ml TNF-α, 1 unit/ml IL-2, and sample. On day 3, 300 μl of the supernatant were replaced with fresh medium containing the same concentration of sample. On day 6, the amount of virus in the supernatant was measured by ELISA for HIV-1 p17 antigen (Eiken Chemical). After sampling, CellTiter96 (MTS) reagent (Promega, Madison, WI) was added to the cells and incubated 2–4 h. The absorbance at 490 nm was determined as a measure of cell proliferation. Basically, coculture assay was referred to (19).

SEAP reporter gene assay. The HIV-1 LTR region (−453 to +208) of pNL4-3 was inserted into the *Hind* III site of pSEAP2-Basic (Clontech, Palo Alto, CA). The hygromycin resistance gene region of pREP7 (Invitrogen, Groningen, The Netherlands) was appended into the *Not* I site of the LTR-SEAP vector. The expression plasmid was transfected into HUT78 and U937 cells using DMRIE-C (Gibco/BRL). Hygromycin resistant cells were cloned in methylcellulose. Clones which expressed SEAP upon induction with TNF-α were selected and named MC141 and CL35, derived from HUT78 and U937, respectively. 5×10^3 MC141 or CL35 cells, seeded in a 96 well plate, were inoculated with 100 μl of PM1000 medium containing 5% FCS, 10 ng/ml TNF-α, and sample. On day 6, SEAP in the supernatant was measured using a chemiluminescence-based detection kit (Roche Molecular Biochemicals).

Transfection assay. pNL4-3 DNA was transfected into MC141 cells using DMRIE-C. Twenty-four hours after transfection, cells were washed and placed in a 48 well plate (2×10^4 cells per well). Cultures were treated as described above for the coculture assay. p17 and SEAP in the supernatant were measured as described above.

Single culture assay. 2×10^4 U1 cells were seeded in a 48 well plate. Cells were treated as described above for the coculture assay. Cell lysates were prepared with 1% Triton X-100 (20), but without an equilibrium centrifugation step.

Blocking the binding between ATF and CD87. Cells were seeded in a 48 well plate as described for the co-culture assay, but no sample was added. The anti-CD87 monoclonal antibody (No. 3936; American Diagnostica, Greenwich, CT) or a mouse IgG control (R&D Systems, Minneapolis, MN) was added at a final concentration of 10 μg/ml. After 2 h incubation, ATF was added at a final concentration of 1.5 ng/ml. Cultures were treated as described above for the coculture assay.

RESULTS AND DISCUSSION

To isolate HIV suppressive factors, we established immortalized $CD8^+$ T cell clones, derived from an HIV-1 infected long-term non-progressor. Culture supernatants from these clones were tested for the ability to suppress HIV-1 replication in an HIV-1 infectious DNA (pNL4-3) transfection assay. HIV-1 viral particles released into the supernatant were detected by ELISA for the HIV-1 p17 antigen. We used the clone that had the highest HIV-1 suppressing activity (clone no. 62) for the remainder of the studies. Preliminarily we assayed the clone no. 62 supernatant for the concentrations of the known HIV-1 suppressor factors by ELISA. Clone no. 62 produced large amounts of β-chemokines and interferon-γ, but no SDF-1, IL-16, or interferon-α. HIV-1 suppressive activity of the clone no. 62 supernatant was still present following acid treatment, which inactivates interferon-γ. β-Chemokines, RANTES, MIP-1α, and MIP-1β, are unlikely to inhibit the NL4-3 laboratory-adapted T-tropic strain of HIV-1 and the transfection assay circumvents the initial viral entry step that is blocked by β-chemokines. Thus, we thought that the activity in the clone no. 62 supernatant was likely due to an unknown soluble factor.

We purified the HIV-1 suppressive activity using eight steps of column chromatography, including SP sepharose, Blue sepharose, Butyl sepharose, hydroxyapatite, Sephacryl S-100 HR, Resource S, and a second round of hydroxyapatite. We followed the suppressor activity throughout the purification process by employing a coculture assay in which cells chronically infected with HIV-1 (TALL-1_{NL4-3}) were incubated with uninfected $CD4^+$ cells (HUT78) at a ratio of 1:4 in the presence of 1 ng/ml TNF-α. The HIV-1 suppressive activity copurified with a single protein that has a molecular mass of 18 kDa as determined by SDS–polyacrylamide gel electrophoresis (Fig. 1A). The 18-kDa band was cut out of the gel and subjected to trypsin digestion, followed by sequencing of distinct peptide fragments. Sequence analysis of three tryptic peptides yielded the sequences KKFG, AXTDTMGRPCLP, and RRPXXYVQXG, respectively. These sequences are identical to sequences in the amino-terminal fragment (ATF) of urokinase-type plasminogen activator (uPA).

Urokinase has been reported to have two isoforms, a high molecular weight form (HMW-uPA) and a low molecular weight form (LMW-uPA). Both forms have intact enzymatic activity. LMW-uPA lacks 135 amino acids found at the amino terminus of HMW-uPA. ATF is this 135 amino acid peptide cleaved naturally during the processing of HMW-uPA to form LMW-uPA, which has no catalytic activity (21). This region contains one EGF-like domain, one kringle domain, and a binding ability to CD87 (the uPA receptor), and has been thought to serve to localize HMW-uPA. To confirm the HIV-1 suppressive activity of ATF, we determined whether ATF from another source could suppress HIV-1 replication. Crude clinical grade uPA that was partially purified from pooled urine of healthy male donors was subjected to Sephacryl S-100 gel filtration chromatography to separate the ATF from HMW-uPA and LMW-uPA. The fractions containing urinary ATF

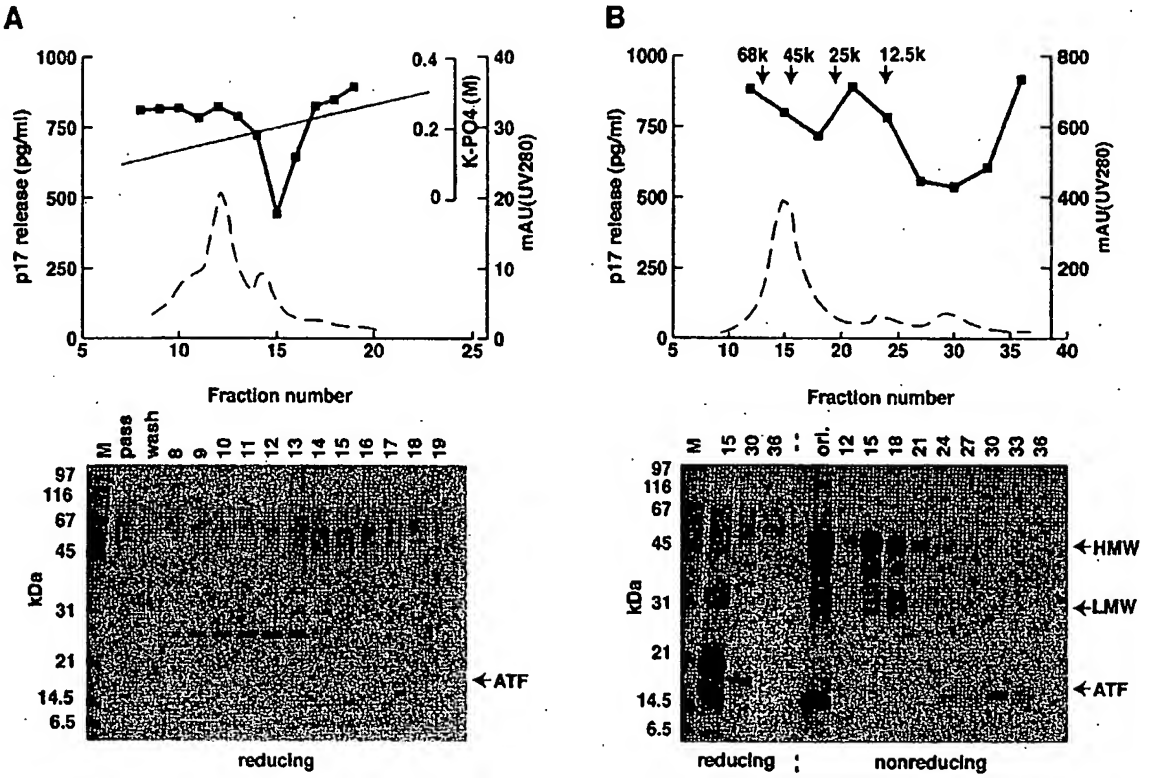

FIG. 1. Purification of HIV-1 suppressive activity. (A) Second round of hydroxyapatite column chromatography of the clone no. 62 supernatant. (B) Sephacryl S-100 chromatography of urinary uPA. HIV-1 suppressive activity of each fraction was assessed in the coculture assay of $TALL_{NL4-3}$ cocultured with HUT78. The extracellular release of HIV-1 p17 antigen (■), potassium phosphate concentration of elution buffer (—), absorbance at 280 nm (- - -). Each fraction was subjected to SDS–PAGE under reducing or nonreducing condition, followed by silver staining.

also showed HIV-1 suppressive activity in the coculture assay (Fig. 1B). In addition, some degradation products with lesser activity were observed. The fractions containing HMW-uPA also suppressed HIV-1 replication but its specific activity was less than that of ATF, even though HMW-uPA contains the sequence of ATF. LMW-uPA, purified further, free from HMW-uPA and ATF had little HIV suppressive activity (data not shown).

A dose dependent inhibition of the extracellular release of HIV-1 p17 antigen by ATF was observed in the coculture assay using either $TALL\text{-}1_{NL4-3}$ cells cocultured with HUT78, or U1 cells cocultured with U937. The maximum inhibition was 50% with 1.5 ng/ml ATF derived from clone no. 62 (Figs. 2A and 2B). In the coculture assays, cell proliferation was not reduced as detected by the tetrazolium salt, MTS. Thus, ATF mediated HIV-1 suppression was not due to cytotoxicity.

To define the point at which ATF inhibits the HIV-1 replication cycle, we constructed $CD4^+$ cell stable transfectants containing the HIV LTR-driven secreted alkaline phosphatase (SEAP) reporter gene. ATF did not affect the secretion of SEAP into the culture medium of either MC141 cells or CL35 cells, which were derived from HUT78 or U937 cells, respectively (Figs. 2C and 2D). Thus, ATF mediated HIV-1 suppression was not due to the inhibition of basal HIV LTR-driven viral transcription or translation.

We then transiently transfected MC141 cells with the infectious HIV-1 DNA, pNL4-3. ATF also suppressed the release of HIV-1 p17 antigen in a dose dependent manner in the transfection assay. The maximum inhibition was 60% using 1.5 ng/ml ATF derived from clone no. 62. In contrast, the secretion of SEAP from the same cells was not suppressed (Fig. 2E). Thus, ATF mediated suppression of HIV-1 was not due to the inhibition of HIV tat-induced transactivation.

The transfection assay includes the early steps of the HIV-1 replication cycle, such as viral entry and reverse transcription, since infectious NL4-3 virions are generated which undergo a second round of infection. Therefore, we used U1 cells in a single culture assay. U1 is a cell line chronically infected with HIV-1, which has been reported to be highly resistant to superinfection by HIV-1 (22), thus eliminating early steps of HIV-1 replication from consideration. ATF also suppressed the release of the HIV-1 p17 antigen into the culture medium of U1 cells. The maximum inhibition

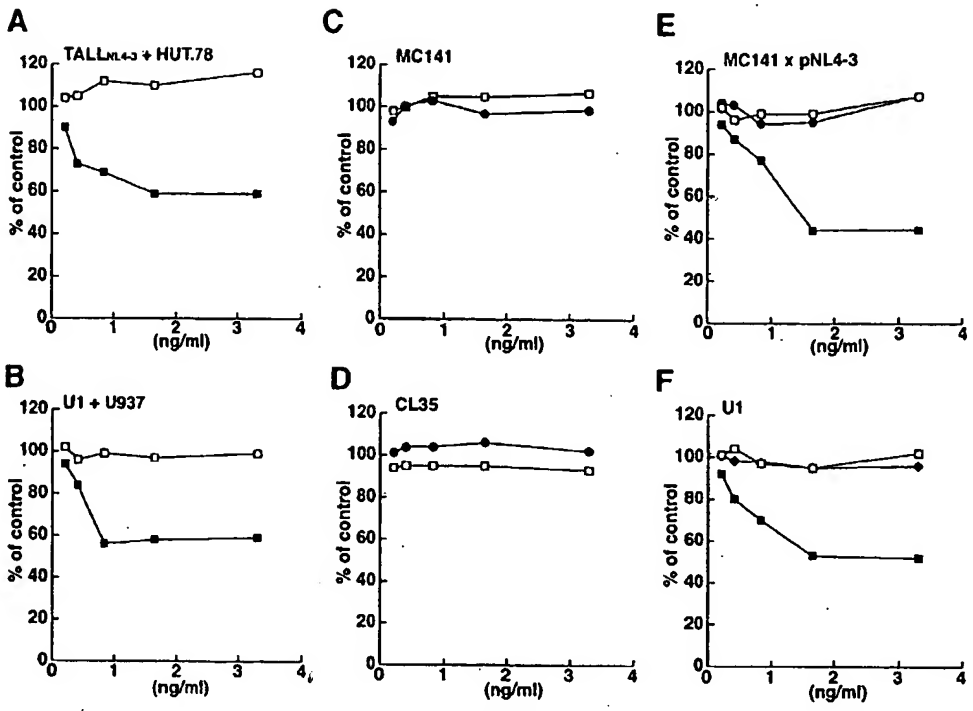

FIG. 2. Dose-dependent effect of ATF derived from clone no. 62 on HIV-1 replication. Various dilutions of purified ATF derived from clone no. 62 (final concentration 0.21–3.3 ng/ml) were tested. (A) In the coculture assay of TALL$_{NL4-3}$ cocultured with HUT78 and (B) U1 cocultured with U937. The extracellular release of HIV-1 p17 (■) and cell proliferation (□). (C) In the SEAP reporter gene assay of MC141, (D) CL35, which were derived from HUT78 and U937, respectively. The secretion of SEAP into the culture medium (●) and cell proliferation (□). (E) In the transfection assay, pNL4-3 infectious DNA was transfected into MC141. The extracellular release of HIV-1 p17 (■), cell proliferation (□), the secretion of SEAP into the culture medium (●). (F) In the single-culture assay of U1. The extracellular release of HIV-1 p17 antigen into the culture medium (■), cell proliferation (□), the accumulation of HIV-1 p17 antigen in cytosol (◆). The same concentrations of buffer for elution of ATF were used as control. The results are representative of at least three independent experiments.

was 55% with 1.5 ng/ml ATF derived from clone no. 62. We also measured the expression level of p17 in the cytoplasm of the U1 cells. The amount of the p17 in the cytosol was not reduced (Fig. 2F). Thus, ATF mediated HIV-1 suppression was due to the inhibition of a late step in the HIV-1 replication cycle, such as assembly or budding of HIV-1 viral particles.

Finally, we tested the relationship between ATF-CD87 binding and the HIV-1 suppressive activity by including an antibody that blocks the interaction between ATF and CD87 in the coculture assays. The anti-CD87 antibody exhibited different effects on HIV-1 replication in different cell types. In T cell lines, both coculture assay of TALL-1$_{NL4-3}$ with HUT78 and transfection assay of HUT78, the anti-CD87 antibody itself suppressed HIV replication either with or without ATF added (Fig. 3A). In promonocytes, U1 cocultured with U937, the anti-CD87 antibody reduced the HIV-1 suppressive activity of ATF (Fig. 3B). In both assays, a control mouse IgG had no effect on HIV-1 replication. These data are consistent with the HIV-1 suppressive activity of ATF.

CD8$^+$ T cell supernatants have been reported to contain suppressive activities for HIV-1 transcription (10, 11, 13, 14). We also detected by the SEAP reporter assay an activity that suppresses HIV-1 transcription in the clone no.62 supernatant, but it could be separated from the ATF-containing fractions during the multi-step chromatography (data not shown).

Unidentified urinary proteins associated with clinical grade human chorionic gonadotropin have been reported active in anti HIV-associated disease, Kaposi's sarcoma (23). Since ATF also existed in urine and its molecular size was close to them, we tested ATF for the proliferation of KS-Y1 neoplastic cells. ATF did not have effect on KS-Y1 proliferation (data not shown).

CD87 is a GPI-anchored protein belonging to the Thy-1/Ly-6 superfamily and is predominantly localized to glycolipid-enriched microdomains called lipid rafts (24). Although CD87 has no cytoplasmic domain, it forms complexes with integrin, caveolin, focal adhesion kinase, and Src family tyrosine kinases in the lipid rafts. ATF-CD87 binding generates a signal for cell adhesion and migration (24–26). Recent reports are highly relevant to our observations. One shows that HIV-1 selectively buds from lipid rafts and that GPI-anchored proteins, such as Thy-1, are incorporated into viral particles (20). The second report shows a relation-

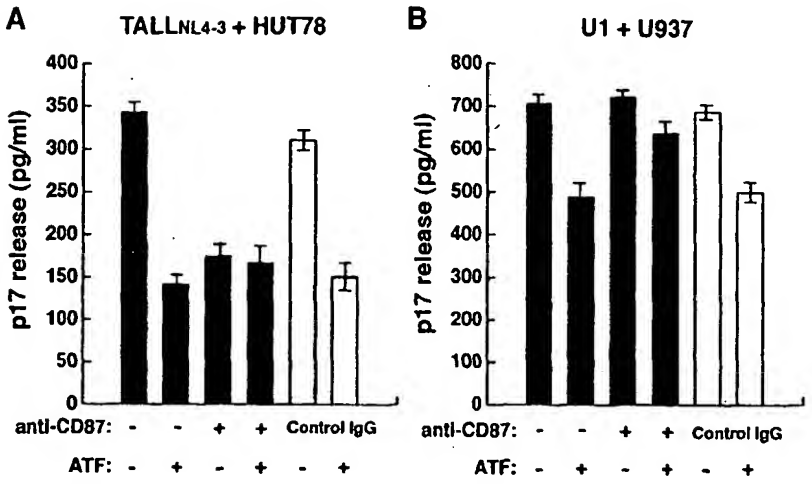

FIG. 3. Effect of anti-CD87 antibody on HIV-1 replication. (A) In the coculture assay of $TALL_{NL4-3}$ cocultured with HUT78 and (B) U1 cocultured with U937. Cells were preincubated with or without CD87 antibody or control mouse IgG for 2 h. Then, ATF was added to cells. The results indicate mean ± SD from three separate experiments.

ship between the level of soluble CD87 in the serum and the survival rates of HIV-1 infected patients (27). Further more, lipid rafts are also required for HIV-1 infection both X4 and R5 strains (28). ATF, or other modulators of the lipid raft environment, may provide alternative options for the treatment of AIDS which could potentially circumvent the problems of drug resistance commonly encountered in current AIDS therapies.

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REFERENCES

1. Levy, J. A., Mackewicz, C. E., and Barker, E. (1996) Controlling HIV pathogenesis: The role of the noncytotoxic anti-HIV response of CD8⁺ T cells. *Immunol. Today* **17**, 217–224.
2. Fauci, A. S. (1996) Host factors and the pathogenesis of HIV-induced disease. *Nature* **384**, 529–534.
3. Tomaras, G. D., and Greenberg, M. L. (2001) CD8+ T cell mediated noncytolytic inhibition of human immunodeficiency virus type 1. *Front. Biosci.* **6**, d575–598.
4. Cocchi, F., Devico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* **270**, 1811–1815.
5. D'Souza, M. P., and Harden, V. A. (1996) Chemokines and HIV-1 second receptors. *Nat. Med.* **2**, 1293–1300.
6. Baier, M., Werner, A., Bannert, N., Metzner, K., and Kurth, R. (1995) HIV suppression by interleukin-16. *Nature* **378**, 563.
7. Zhou, P., Goldstein, S., Devadas, K., Tewari, D., and Notkines, A. L. (1997) Human CD4+ cells transfected with IL-16 cDNA are resistant to HIV-1 infection: Inhibition of mRNA expression. *Nat. Med.* **3**, 659–664.
8. Maciaszek, J. W., Parada, N. A., Cruikshank, W. W., Center, D. M., Kornfeld, H., and Viglianti, G. A. (1997) IL-16 represses HIV-1 promoter activity. *J. Immunol.* **158**, 5–8.
9. Pal, R. Garzino-Demo, A., Markham, P. D., Burns, J., Brown, M., Gallo, R. C., and DeVico, A. L. (1997) Inhibition of HIV-1 infection by the β-chemokine MDC. *Science* **278**, 695–698.
10. Mackewicz, C. E., Blackbourn, D. J., and Levy, J. A. (1995) CD8⁺ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. *Proc. Natl. Acad. Sci. USA* **92**, 2308–2312.
11. Barker, E. (1999) CD8⁺ cell-derived anti-human immunodeficiency virus inhibitory factor. *J. Infect. Dis.* **179**, S485–488.
12. Moriuchi, H., Moriuchi, M., Combadiere, C., Murphy, P. M., and Fauci, A. S. (1996) CD8⁺ T-cell-derived soluble factor(s), but not β-chemokines RANTES, MIP-1α, and MIP-1β, suppress HIV-1 replication in monocyte/macrophages. *Proc. Natl. Acad. Sci. USA* **93**, 15341–15345.
13. Chen, C., Weinhold, K. J., Bartlett, J. A., Bolognesi, D. P., and Greenberg, M. L. (1993) CD8⁺ T lymphocyte-mediated inhibition of HIV-1 long terminal repeat transcription: A novel antiviral mechanism. *AIDS Res. Hum. Retroviruses* **9**, 1079–1086.
14. Copeland, K. FT., Mckay, P. J., and Rosenthal, K. L. (1995) Suppression of activation of the human immunodeficiency virus long terminal repeat by CD8⁺ T cells is not lentivirus specific. *AIDS Res. Hum. Retroviruses* **11**, 1321–1326.
15. Kannagi, M., Masuda, T., Hattori, T., Kanoh, T., Nasu, K., Yamamoto, N., and Harada, S. (1990) Interference with human immunodeficiency virus (HIV) replication by CD8⁺ T cells in peripheral blood leukocytes of asymptomatic HIV carriers in vitro. *J. Virol.* **64**, 3399–3406.
16. Tsuchie, H., Detorio, M. A., Hossain, M. M., Tesfamariam, T., Trickett, A., Lam-Po-Tang, P. R., Yamada, O., Ichimura, H., Dwyer, J. M., and Kurimura, T. (1997) Suppression of HIV replication in vitro by CD8⁺ T-cells from HIV-infected and HIV-seronegative individuals. *Int. J. STD AIDS* **8**, 307–310.
17. Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obaishi, H., Matsuura, Y., and Takai, Y. (1997) Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. *J. Biol. Chem.* **272**, 3875–3878.
18. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. (1986) Production of acquired immu-

nodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**, 284–291.

19. Murakami, T., Nakashima, H., Ito, M., and Yamamoto, N. (1992) A novel coculture system for evaluating anti-HIV drugs. *AIDS* **6**, 1279–1285.
20. Nguyen, D. H., and Hildreth, J. E. K. (2000) Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* **74**, 3264–3272.
21. Stoppelli, P. M., Corti, A., Soffientini, A., Cassani, G., Blasi, F., and Assoian, R. K. (1985) Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc. Natl. Acad. Sci. USA* **82**, 4939–4943.
22. Chen, B. K., Saksela, K., Andino, R., and Baltimore, D. (1994) Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. *J. Virol.* **68**, 654–660.
23. Lanardi-Iskandal, Y., Bryant, J. L., Blattner, W. A., Hung, C. L., Flamand, L., Gill, P., Hermans, P., Birken, S., and Gallo, R. C. (1998) Effects of a urinary factor from women in early pregnancy on HIV-1, SIV and associated disease. *Nat. Med.* **4**, 428–434.
24. Koshelnick, Y., Ehart, M., Stockinger, H., and Binder, B. R. (1999) Mechanisms of signaling through urokinase receptor and the cellular response. *Thromb. Haemost.* **82**, 305–311.
25. Ossowski, L., and Aguirre-Ghiso, J. A. (2000) Urokinase receptor and integrin partnership: Coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* **12**, 613–620.
26. Preissner, K. T., Kanse, S. M., and May, A. E. (2000) Urokinase receptor: A molecular organizer in cellular communication. *Curr. Opin. Cell Biol.* **12**, 621–628.
27. Sidenius, N., Sier, C-F. M., Ullum, H., Pedersen, B. K., Lepri, A. C., Blasi, F., and Eugen-Olsen, J. (2000) Serum level of soluble urokinase-type plasminogen activator receptor is a strong and independent predictor of survival in human immunodeficiency virus infection. *Blood* **96**, 4091–4095.
28. Mañes, S., Real, G. D., Lacalle, R. A., Lucas, P., Gómez-Moutón, C., Sánchez-Palomino, S., Delgado, R., Alcamí, J., Mira, E., and Martínez-A, C. (2000) Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. *EMBO Rep.* **1**, 190–196.

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A Role for Urokinase-Type Plasminogen Activator in Human Immunodeficiency Virus Type 1 Infection of Macrophages

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Urokinase-type plasminogen activator (uPA), a proteinase which activates plasminogen by cleaving at -CPGR ↓ V-, was shown to cleave the V3 loop in recombinant gp120 of human immunodeficiency virus type 1 (HIV-1) IIIB and MN strains, as well as a synthetic, cyclized peptide representing the clade B consensus sequence of V3. Proteolysis occurred at the homologous -GPGR ↓ A-, an important neutralizing determinant of HIV-1. It required soluble CD4 and was prevented by inhibitors of uPA but not by inhibitors of likely contaminating plasma proteinases. It was accelerated by heparin, a known cofactor for plasminogen activation. In immune capture experiments, tight binding of uPA to viral particles, which did not depend on CD4, was also demonstrated. Active site-directed inhibitors of uPA diminished this binding, as did a neutralizing antibody to V3. Addition of exogenous uPA to the laboratory-adapted IIIB strain of HIV-1, the macrophage-tropic field strains JR-CSF and SF-162, or a fresh patient isolate of indeterminate tropism, followed by infection of macrophages with the various treated viruses, resulted in severalfold increases in subsequent viral replication, as judged by yields of reverse transcriptase activity and p24 antigen, as well as incorporation, as judged by PCR in situ. These responses were reversible by inhibitors or antibodies targeting the proteinase active site or the V3 loop. We propose that uPA, a transcriptionally regulated proteinase which is upregulated when macrophages are HIV infected, can be bound and utilized by the virus to aid in fusion and may be an endogenous component that is critical to the infection of macrophages by HIV-1.

Entry of human immunodeficiency virus type 1 (HIV-1) into primate cells occurs through interactions at several levels between viral envelope proteins gp120 and gp41 and the host cell membrane. After binding of gp120 to the receptor CD4 (32), structural changes that permit an N-terminal hydrophobic peptide of gp41 to penetrate the lipid bilayer, mediating fusion, are believed to occur (11, 36). The site of origin of these changes is obscure, but strongly implicated is the crown of the third variable (V3) loop region of gp120, which is distinct and remote from the domains involved in CD4 binding and contains the amino acid sequence -GPGRA- or -GPGRV- (12, 28). This sequence is highly conserved among (and, indeed, defines) clade B strains (35) of HIV-1, despite the mutability of the surrounding amino acids which earns the region its name. An important neutralizing determinant for monoclonal or patient-derived antibodies to gp120 also resides in -GPGR-, underscoring its importance in viral behavior. -GPGRA- and its counterparts in other clades, frequently -GPGQA- and -GPGQV-, adopt a type II β-turn structure (13) that remains accessible to antibodies in the assembled viral envelope (33). Variants of HIV-1 altered in -GPGRA/V- can escape antibody neutralization (23): some are not replication competent and, specifically, not fusogenic (39), despite the fact that binding to CD4 persists unimpaired (4, 11, 43).

As predicted from its homology to -GPCRA-, a sequence found at the binding sites of proteinase inhibitors such as trypstatin and inter-α-trypsin inhibitor (18, 24, 34), gp120 at the V3 loop is also a potential substrate or binding site for certain secreted or cellular proteinases (3, 7, 17, 47). Given the strong homology of -GPGRA/V- to the activation sequence -CPGRV- in human plasminogen, we investigated whether gp120 is recognized by urokinase-type plasminogen activator (uPA), a receptor-bound proteinase produced by macrophages as part of their tissue-invasive machinery and inflammatory response. We have found that not only does uPA bind and cleave gp120 at V3, but it is also responsible for significant increases in the infectivity of HIV-1 toward these cells.

(This work was presented in preliminary form [16] at the May 1994 meeting of the American Federation for Clinical Research/American Society for Clinical Investigation/Association of American Physicians in Baltimore, Md.)

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MATERIALS AND METHODS

Cleavage of gp120. Recombinant full-length, fully glycosylated gp120 of HIV-1 IIIB strain (Intracel Corp.) or HIV-1 MN strain (Agmed) was incubated at 20 μg/ml with two-chain recombinant uPA (ruPA; 600 IU or ~6 μg/ml; Abbott Laboratories), recombinant soluble CD4 (rsCD4; 5 μg/ml; DuPont-NEN), and heparin (25 U/ml), which increases uPA proteolytic activity (45). When necessary, sCD4 was pretreated with DEGR-CMK (1,5-dansyl-Glu-Gly-Arg-chloromethyl ketone dihydrochloride; 5 μM for 30 min) to inactivate a contaminating proteinase derived from the CHO cell of origin (7). Reagents tested for the ability to inhibit cleavage of gp120 included (i) murine immunoglobulin G1 (IgG1) antibody to the uPA catalytic site (antibody 394; American Diagnostica); (ii) IgG1 antibody to the uPA receptor-binding domain (antibody 3921; American Diagnostica); (iii) endothelial cell PAI-1 (American Diagnostica); (iv) α_2-plasmin inhibitor (American Diagnostica); (v) a neutralizing monoclonal antibody (antibody 9205; DuPont-NEN) to an epitope in V3 of HIV-1 IIIB spanning the cleavage site, GPGR ↓ A (9); and (vi) a nonneutralizing antibody (antibody IIIB-V3-01) to the sequence IGKIGNMRQ four residues C terminal to the putative cleavage site (27). Samples of gp120 denatured and reduced in 5 mM EDTA–40 mM imidazole–1 M glycerol–0.05% bromophenol blue–1% sodium dodecyl sulfate (SDS)–5% 2-mercaptoethanol (pH 6.4) were electrophoresed in 10% polyacrylamide (26). After the gels were washed in 20% methanol–25 mM Tris–0.2 M glycine, proteins were transferred (Pharmacia Novablot) to Immobilon P (Millipore). Blots were blocked overnight in 5% nonfat milk–0.1 M NaCl–20 mM Tris (pH 7.5), then incubated with sheep antibody to gp120 C5 region (BioDesign International)–2 μg of IgG per ml for 4 h, followed by alkaline phosphatase-conjugated donkey anti-sheep IgG (1/30,000) absorbed with rodent

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and other sera (Jackson Immunoresearch), and developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP)–nitroblue tetrazolium (NBT) premixed substrate (Kirkegaard & Perry Laboratories).

High-pressure liquid chromatography (HPLC). The 35-residue, disulfide-bonded consensus sequence for V3 loop peptide of HIV-1 clade B (Intracel) was incubated at 1.0 mg/ml with ruPA (600 U/ml) or human thrombin (10 μg/ml) in phosphate-buffered saline (PBS) for up to 6 h. No sCD4 or heparin was present. Samples of 5 μg, reduced with 0.25 M dithiothreitol, were injected via a cartridge precolumn (Brownlee) into a 5-cm-long, 4.6-mm-diameter, 300-Å (30-nm)-pore-size LC-18 reverse-phase column (Supelcosil). Elution was with a 0 to 60% CH_3CN gradient in 0.1% phosphoric acid–0.01 mM dithiothreitol–water. Effluent absorbance was monitored at 210 nm. The marker for proteolysis was the 18-residue peptide that would derive C terminally from cleavage at -GPGR ↓ A- and subsequent reduction. This peptide was synthesized by standard resin-based methodology and verified by sequencing.

Cell culture. Mononuclear cells of healthy uninfected donors were obtained, with informed consent, from 200 ml of heparinized blood by centrifugation through Ficoll-Hypaque (Pharmacia) (5). After collection from the interface, the cells were washed three times in RPMI 1640 medium (Whittaker), transferred to flasks, and incubated under humidified 5% CO_2. Nonadherent cells were removed after 20 h by replacing with RPMI 1640–20% fetal bovine serum with endotoxin <30 pg/ml (Sigma)–5% autologous heat-inactivated human serum, glutamine, and antibiotics. On day 4, adherent macrophages were lifted by scraping, washed, resuspended, and replated in 12- or 24-well plates at 10^6 per well. They were >90% viable by trypan blue exclusion and >98% positive for monocyte nonspecific esterase. Analysis by flow cytometry (FACStar Plus; Becton Dickinson) typically indicated 99% positivity for LeuM5 plus HLA-DR; >96% positivity for CD14 (monocytes); and ≤1% positivity for CD3 (pan-T), CD4 (T4), CD19 (pan-B), CD15 (neutrophils), or CD56 (natural killer cells). At 2 h, aliquots of HIV-1 IIIB from H9 lymphoblastoma cells (40) were added at 5,000 to 20,000 cpm of reverse transcriptase (RT) activity (19) after treatment of some aliquots with ruPA (600 U/ml) for 4 h at 37°C, with or without antibody 394 (50 μg/ml) or PAI-1 (800 U/ml). The virus was washed free of unbound uPA, etc., before infection. Control cultures were infected with untreated HIV, up to 100,000 cpm of RT (50% tissue culture infective dose, ~8×10^4), or sham infected with medium alone. During culture, the medium was replaced every 4 days. Samples from alternate days were assayed for p24 antigen by capture enzyme-linked immunosorbent assay (ELISA; DuPont-NEN) and for RT by a colorimetric method (46).

Other strains. In experiments investigating additional viral strains, HIV-$1_{JR\text{-}CSF}$ and HIV-$1_{SF\text{-}162}$ were cultured for 5 days in phytohemagglutinin-stimulated peripheral blood lymphocytes in the presence of interleukin-2 (Boehringer Mannheim) (20) and quantified by RT activity (19). Patient isolate 5056 was recovered from culture supernatants of the patient's peripheral blood lymphocytes cocultured with normal donor lymphocytes at a ratio of 1:5 (20). Aliquots ranging between 160 and 5,000 cpm of RT activity were treated at a fixed uPA concentration (600 U/ml) and used to infect 10^6 macrophages per aliquot as described above.

PCR in situ. The procedure was performed as described previously (37). Macrophages lifted by scraping 72 h after infection with HIV-1 IIIB were fixed in 10% formaldehyde–PBS for 5 min. Aliquots of 10^5 cells were spun onto siliconized slides and ethanol fixed, treated for 5 min with proteinase K (20 μg/ml), and then subjected to 24 cycles of PCR (2 min, 55°C; 1 min, 94°C; 1 min, cooling; 5 min, 61 to 57°C) with *Taq* polymerase (0.18 U/ml; Cetus) in 10 mM Tris–50 mM KCl–1.5 mM $MgCl_2$ containing 0.2 mM digoxigenin-labeled deoxynucleoside triphosphates and primer SK19 of *gag* (38). Amplified HIV-1 DNA was detected with the Genius system (Boehringer Mannheim) as instructed by the manufacturer. Cells were counterstained with nuclear fast red. Nuclei stained blue-black with NBT were counted by two observers unaware of the applicable conditions, and the values were averaged.

Association of uPA with HIV-1 virions. ruPA (500 U/ml) was added to HIV-1 IIIB (7.5×10^6 cpm of RT per ml) in the presence or absence of PAI-1 (500 U/ml), neutralizing monoclonal antibody 9205 to the V3 loop, or antibody 394 to the uPA catalytic site (50 μg/ml). After incubation for 1 h, the viral pellet was recovered, washed twice by resuspension, dissociated in SDS, electrophoresed, and immunoblotted as described above. The blot was probed with goat anti-uPA and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma) and developed with BCIP-NBT.

Immunocapture of HIV-1 by antibody to uPA. HIV-1 IIIB (60 cpm of RT activity) was incubated with 50 U of ruPA in 100 μl of PBS for 4 h at 37°C. Goat anti-human uPA (antibody 398; 25 ng; American Diagnostica) was added, the mixture was incubated for 1 h at ambient temperature, and then 10 ng of rabbit anti-goat IgG heavy and light chains (Sigma) was added for a further 1 h. Complexes containing rabbit IgG were captured on 400 μl of staphylococcal protein A-agarose, washed with 40 volumes of PBS, and solubilized in 5% Triton X-100–PBS before assay for HIV-1 p24 antigen by ELISA. Data were corrected for nonspecific binding of rabbit antibody in the absence of goat IgG. Binding was also measured after treatment of uPA with DEGR-CMK (up to 1.0 μM) or PAI-1 (200 IU/ml).

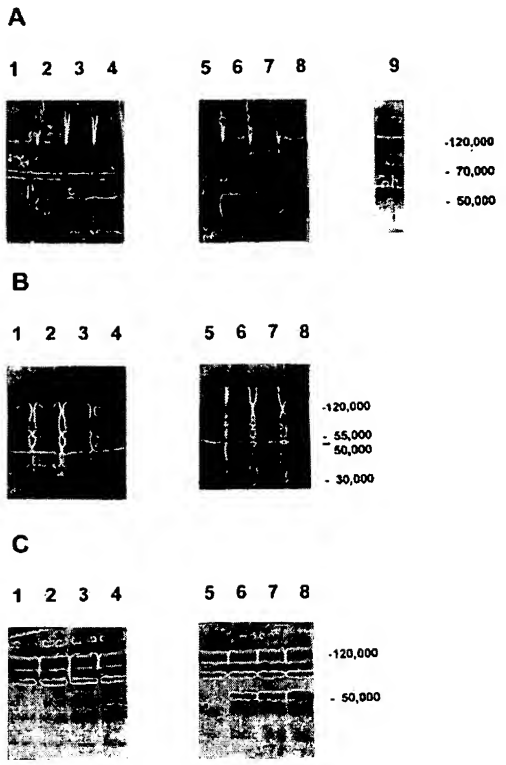

FIG. 1. Cleavage of gp120 by uPA and prevention by specific uPA inhibitors. (A) Cleavage of gp120 by uPA (600 U/ml; lanes 1 to 4), or thrombin, (2.5 μg/ml; lanes 5 to 8) at 0, 2, 4, and 6 h. The 70-kDa cleavage product could be visualized by immunoblotting with human rather than sheep antibody (lane 9). Approximate molecular weights are relative to prestained standards. (B) Cleavage by uPA at 0, 2, 4, and 6 h in the presence of antibody to the uPA catalytic site (lanes 1 to 4) or antibody to the receptor-binding domain (lanes 5 to 8). (C) Cleavage by uPA at 0, 2, 4, and 6 h in the presence of PAI-1 (lanes 1 to 4) or human α_2-plasmin inhibitor (lanes 5 to 8). Residual cross-reactivity of the detecting antibodies with albumin in ruPA, and with heavy and light chains of murine antibodies, accounts for immunopositive bands at ~70, ~55, and 30 kDa.

RESULTS

Specific cleavage of HIV-1 gp120. When recombinant glycosylated gp120 of HIV-1 IIIB was incubated with uPA, a 50-kDa fragment was detectable on Western blots (immunoblots) with sheep antibody to the C terminus of gp120, which increased in intensity over time (Fig. 1A, lanes 1 to 4) and resembled a fragment obtained by cleavage with thrombin (7) (lanes 5 to 8). The N-terminal, 70-kDa fragment of gp120 was visible when similar blots were probed with IgG from a donor with high-titer antibody to gp120 (lane 9). Similar 50- and 70-kDa fragments were generated when rgp120 of HIV-1 MN was incubated with uPA or thrombin (not shown).

A murine IgG1 monoclonal antibody to the catalytic site of uPA blocked cleavage over time (Fig. 1B, lanes 1 to 4), whereas IgG1 antibody to the receptor-binding domain of uPA, with no effect on catalytic activity in the absence of the uPA receptor, did not (Fig. 1B, lanes 5 to 8). Similarly, the endothelial plasminogen activator inhibitor PAI-1 inhibited proteolysis (Fig. 1C, lanes 1 to 4), but α_2-plasmin inhibitor had no effect (Fig. 1C, lanes 5 to 8). Antithrombin, an inhibitor of thrombin and related proteinases, also had no effect on cleavage, nor did Ca^{2+} or EDTA (data not shown). Therefore, proteolysis apparently occurred without the intervention of

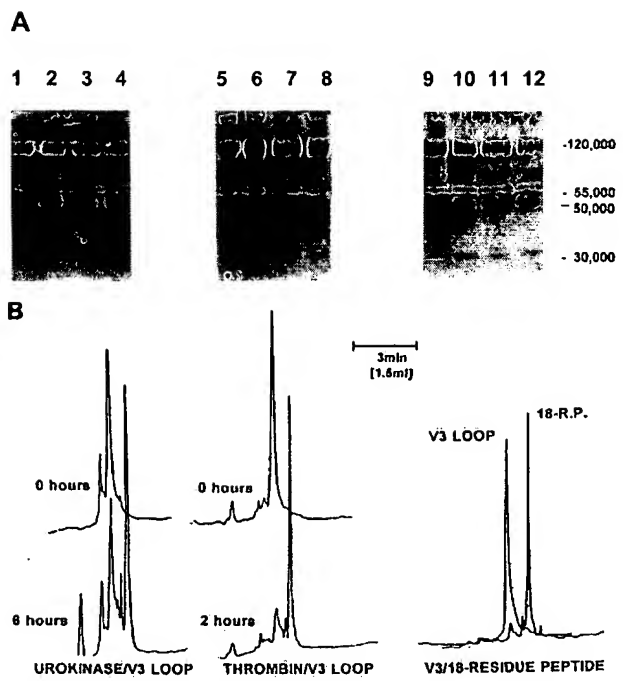

FIG. 2. Identification of the site of cleavage as the V3 loop. Lanes 1 to 4, control without antibody; lanes 5 to 8, samples over time with neutralizing antibody spanning the cleavage site; lanes 9 to 12, samples with nonneutralizing antibody to V3. Residual cross-reactivity of the detecting antibodies with albumin in ruPA. and with heavy and light chains of murine antibodies, accounts for immunopositive bands at ~70, ~55, and 30 kDa. (B) Separation of peptides generated during cleavage of V3 loop by uPA or thrombin. Effluent absorbance at 210 nm from a C_{18} reverse-phase HPLC column is shown. 18-R.P., the marker for proteolysis, is the C-terminal peptide that would derive from cleavage and reduction of V3. This peptide lagged 0.75 ml behind intact V3 loop when a mixture of the two was chromatographed (far right). Incubation of V3 with thrombin (center) or uPA (left) generated cleavage product over time.

plasmin or other plasma-derived proteinases; it resulted from uPA as such.

Site of cleavage in gp120. To verify the site of proteolysis, the effects of monoclonal antibodies to epitopes in V3 were compared. Relative to nonimmune murine IgG1 (Fig. 2A, lanes 1 to 4), a neutralizing monoclonal IgG1 antibody (9) to an epitope spanning -GPGR- prevented cleavage (Fig. 2A, lanes 5 to 8), whereas a nonneutralizing IgG1 antibody to a flanking sequence close by (27) did not (Fig. 2A, lanes 9 to 12). This result shows that neutralization occurs specifically at the potential cleavage site. Furthermore, when the synthetic, 35-residue cyclized V3 peptide was incubated as such with uPA or thrombin, cleavage products generated by either proteinase had the same chromatographic characteristics as the 18-residue C-terminal product of cleavage at -GPGR ↓ A- (Fig. 2B). This reaction occurred in the absence of CHO cell-derived recombinant reagents, eliminating the CHO cell protease known to cleave gp120 (7) as responsible for V3 cleavage in our experiments.

Effects of uPA on viral infectivity and replication. Mature monocyte-derived macrophages readily increase expression of uPA in response to hematopoietic or inflammatory stimuli (14, 29). Therefore, the possibility that uPA proteolytic activity and infection of macrophages by HIV-1 are related was investigated. Blood monocytes from healthy donors were isolated and allowed to adhere and differentiate in culture. After cellular homogeneity was confirmed by flow cytometry, the macrophages were infected with HIV-1 IIIB which had been previously cultured in H9 lymphoblastoma cells (40) and preincubated (without addition of sCD4) in aliquots of 5,000 cpm of RT with pyrogen-free ruPA (600 U/ml) immediately prior to infection. This plan was designed to avoid any effects of uPA on macrophages that might alter viral behavior, as well as interference by proteinase inhibitors in serum-supplemented media. The infectivity of the proteinase-treated virus was determined in three ways: (i) RT assay of culture supernatants (46) and (ii) p24 antigen assay, both of which assessed production of new virions 7 to 9 days after infection; and (iii) PCR in situ (37), which indicated the proportion of cells containing proviral DNA 3 days after infection. Infection was at a low 50% tissue culture infectious dose of 2.2×10^4, at which, without prior uPA treatment, the yield of RT was at or near the background for uninfected media (Fig. 3A). In contrast, after exposure of HIV-1 to uPA, the RT yield increased to >0.6 absorbance units; this uPA-dependent increase was attenuated 100% on day 7 and ~70% on day 9 when antibody to the uPA active site was present during the preincubation step (Fig. 3A).

In this and a second identical experiment, undetectable replication was increased to these highly significant levels by pretreatment of the HIV-1 inoculum with uPA, as shown (Fig. 3A). In six more experiments, in which there was some baseline

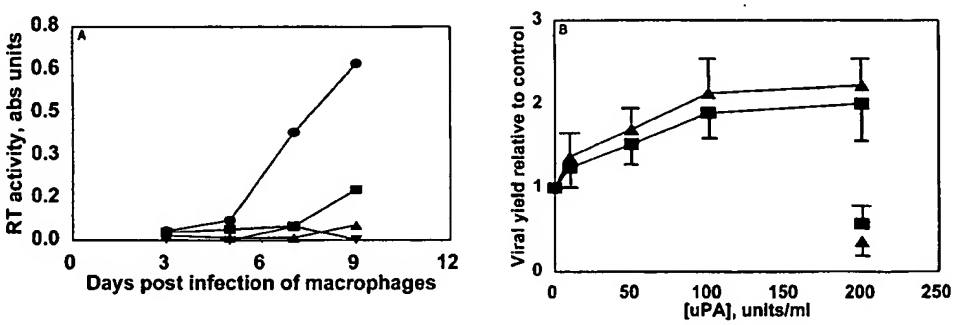

FIG. 3. Increased yield of HIV-1 from macrophages after treatment of the inoculum with uPA. (A) Yield of RT activity over time from a culture infected with HIV-1 that was (●) or was not (▲) pretreated with uPA. ■, inoculum treated in the presence of antibody to uPA; ▼, baseline activity from uninfected cells. This experiment is typical of eight representing different donors, with each analysis performed in duplicate or triplicate. Statistical analysis is given in the text. abs, absorbance. (B) Dose response to uPA concentrations of between 0 and 200 U/ml. The input level of HIV-1 was 10,000 to 20,000 cpm of RT activity per aliquot. Data shown are means from six experiments, each analysis performed in duplicate. ■, RT activity; ▲, p24 antigen; solitary points, virus treated with uPA (200 U/ml) in the presence of PAI-1 (800 U/ml).

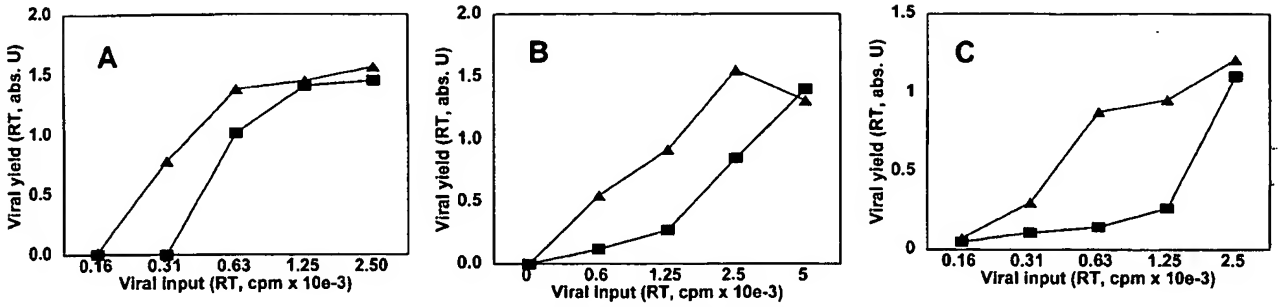

FIG. 4. Responses of field strains of HIV-1 to pretreatment with uPA at a fixed concentration over a range of HIV-1 inoculum size (■) relative to untreated HIV-1 (▲). Each experiment was performed twice, and one example of each is shown. (A) HIV-$1_{JR\text{-}CSF}$; (B) HIV-1_{SF162}; (C) a fresh isolate, 5056. abs., absorbance.

infection without exposure of virus to proteinase, RT activity after uPA treatment was increased from a minimum of 5-fold to a maximum of 100-fold ($P < 0.0001$ by pairwise t test).

By PCR in situ analysis (six experiments), the number of nuclei staining for incorporated viral DNA with the *gag* SK19 probe (38) on day 3 postinfection, before replication was detectable, increased from 26.1% ± 4.6% to 78.1% ± 1.4% (means ± standard errors of the means; $p = 0.0001$ by pairwise t test). This was equal to the percentage of cells (75.6% ± 3.2%) containing proviral DNA in control cultures that were infected maximally with 20 times more HIV-1 IIIB. Theoretically these results could be affected by the presence of positively stained but unintegrated, circular forms of HIV-1 DNA. However, it seems unlikely that a change in the number of these forms could account solely for the effects of uPA, acting extracellularly on the virion or macrophages, particularly as upregulation is most pronounced at low viral input levels (see below).

In additional experiments, it was confirmed that the mean increase in viral yield was dose dependent with respect to uPA (Fig. 3B). In six cultures, mean yields of RT activity rose as uPA concentrations ranged between 0 and 200 U/ml, while p24 antigen increased concomitantly. Both p24 and RT were decreased to levels at or below baseline in the presence of PAI-1 (500 U/ml), confirming that the uPA was responsible for the enhancement of infectivity. Moreover, this upregulation, like cleavage, occurs independently of plasminogen activation, as levels of plasmin(ogen) in the ruPA are very low indeed, >12 to 36 pg per mg of uPA. Also, α_2-plasmin inhibitor did not reverse the increase in infectivity caused by uPA (data not shown). However, it cannot be entirely ruled out that the viral particle carries with it traces of plasminogen from prior exposure to serum-supplemented culture medium.

The HIV-1 used in the preceding experiment was the laboratory-adapted IIIB strain, with its possible idiosyncrasies caused by multiple passaging and the insertion of two amino acids into V3 adjacent to the cleavage site (35). Therefore, we sought to determine whether uPA would influence infectivity of HIV-1 of strains deriving from two field isolates, SF162 (6) and JR-CSF (25), from clade B and minimally passaged in a primary lymphocyte culture, as well as a fresh patient isolate treated in a similar manner. It was found that these strains displayed different intrinsic infectivities toward macrophages when the inoculum size was based on RT activity. Therefore, the inoculum of HIV-1 used was titrated from 0 to 5,000 cpm of RT activity, with or without prior treatment at a fixed concentration of uPA, 600 U/ml. The results are shown in Fig. 4 as a function of viral input. One of two experiments performed with each of three isolates in different donor cells is shown (Fig. 4A, B, and C). The data are corrected for the low background activity of uninfected cells (<0.1 absorbance units). As for IIIB, levels of viral input had to be decreased well below maximum infection for enhancement by uPA to be the most striking. Under these conditions, the yield of RT was increased severalfold in response to uPA in all three strains. This increase was greatest at viral inputs of between 300 and 1,250 cpm of RT, depending on the strain. Taken together, these results indicate that upregulation of infectivity by uPA is by no means exclusive to the laboratory-adapted IIIB strain but extends to three others that had been minimally passaged.

Binding of uPA to HIV-1 particles. The rates of cleavage of gp120 versus the kinetics of upregulation of infectivity are widely different, and a possible problem with proposing uPA as an agent for obligatory cleavage is that, under the conditions described here, the rate is quite slow. One explanation may be that the in vitro system lacks physiologic components that would considerably enhance the kinetics of plasminogen activation, importantly the uPA receptor (10, 30). Another possibility is that the uPA active site serves a binding rather than catalytic function, with cleavage occurring only incidentally and at a slower pace. Therefore, the ability of HIV-1 gp120 to bind uPA was investigated.

In experiments in which uPA was added to a high-titer suspension of HIV-1 IIIB (7.5×10^6 cpm of RT activity per ml), virus recovered from the suspension by centrifugation carried virtually all of the uPA detectable by immunoblotting. This apparent association of uPA with viral particles was prevented equally well by antibody to the uPA catalytic site, PAI-1, or neutralizing antibody to the V3 loop (Fig. 5A).

Mindful of the possibility that cellular debris contributed to this apparent binding, we investigated the phenomenon further in immune capture experiments not dependent on centrifugation. When uPA, goat antibody to uPA, a rabbit anti-goat IgG, and staphylococcal protein A-agarose were added sequentially to suspensions of HIV-1, the complexes recovered by elution from protein A at low pH contained 79.6 ± 7.3 pg (mean ± standard deviation) of p24 antigen per ml (Fig. 5B). This was significantly more than the mean amount of p24 recovered from complexes containing no exogenous uPA (seven experiments; $P < 0.0001$ by pairwise t test). However, the latter did contain 16.5 ± 3.9 pg of p24 antigen per ml compared with controls containing no goat anti-uPA antibody in which p24 antigen was undetectable (Fig. 5B), suggesting a possible association of endogenous uPA with viral particles. Moreover, the association between uPA and p24 was decreased, in a dose-dependent fashion, by the uPA active site inhibitor DEGR-CMK at concentrations of up to 1.0 μM ($n = 6$; $P = 0.003$) or by PAI-1 at 200 U/ml ($n = 4$; $P = 0.048$) (Fig. 5B).

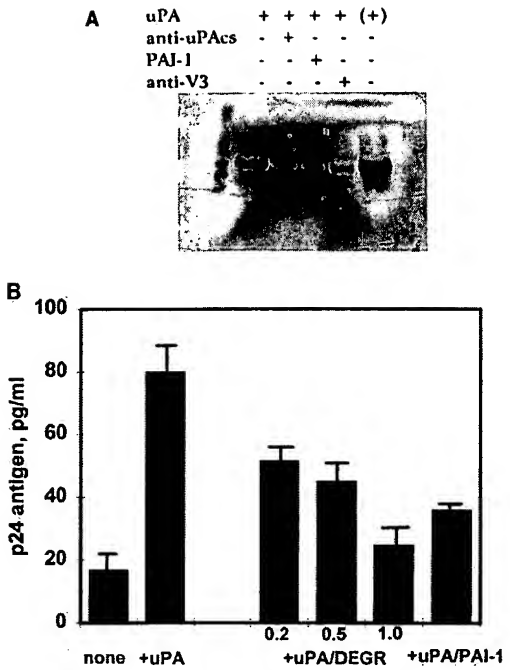

FIG. 5. Association of uPA with viral particles and prevention of association by active site-directed inhibitors. (A) HIV at high titer was mixed with uPA with or without various inhibitors as indicated above the blot and recovered by centrifugation. uPA with the same mobility as the parent ruPA (far right) was visualized by immunoblotting (see Materials and Methods). Anti-uPA cs, antibody to the uPA catalytic site. (B) HIV at low titer was mixed with uPA with or without DEGR-CMK (at the concentrations [micromolar] indicated at the bottom) or PAI-1, recovered by immunocapture with anti-uPA antibody, and quantified in an ELISA from the content of p24 antigen. Data are shown as means ± standard deviations (bars) and are corrected for assay background, determined from *p*-nitrophenol generation in the absence of primary antibody to uPA.

Therefore, by several criteria, the binding of uPA to HIV-1 particles appears to be specific and mediated through interaction between the exposed V3 loop of the virus and the proteinase active site.

DISCUSSION

We have shown that uPA binds tightly to HIV-1 particles via gp120 at V3 and can also cleave gp120 of IIIB and MN strains within the V3 loop in the presence of sCD4. These reactions are prevented with a variety of inhibitors targeted to either side of the interaction (uPA or viral envelope), whereas inhibitors of potential contaminating blood proteinases are without effect. uPA also causes a large, dose-dependent increase in the infectivity of both laboratory-adapted and field strains of HIV-1. Neutralizing antibody targeting V3 of IIIB prevents this effect also, as well as preventing the proteolysis of V3 and infection. As uPA is a critical enzyme in the inflammatory response and in the invasive properties of macrophages, we believe that its interaction with the viral envelope is an important element in the infection of activated macrophages by HIV-1.

Although the sites of interaction between virus and uPA have been identified, the extent and type of the interaction are still somewhat in doubt, as it is not known whether cleavage as such is obligatory for fusion. Given the disparity in rate between binding and proteolysis of gp120 by uPA, we propose as a working hypothesis that the binding of uPA to virus particles, which probably occurs independently of CD4, may be the decisive interaction. This would be consistent with observations that other cellular proteinases, such as cathepsin G and surface proteinases of MOLT-4 cells, recognize and bind to gp120 or peptides derived from the -GPGRA/V- sequence (3, 17, 42). Given the binding interaction, it is conceivable that uPA acquired from the vicinity of the macrophage is carried with viral particles into other surroundings and to other cells. The roles of this mode of transport in infection and in cellular tropism are currently being investigated.

Although the susceptibility of gp120 to proteases is not a new observation, it has not previously been linked to altered viral behavior in HIV-1 or to a pathway that would particularly assist in fusion of HIV-1 with macrophages. The phenomenon of increased infectivity after treatment with proteinases was seen with another retrovirus, however (1), while intracellular cleavage of gp160, the gp120 precursor, by eukaryotic proteinases such as furin or its homologs (8) is known to be required for competence in subsequent fusion (2). Proteolytic cleavage of spike proteins by cellular proteinases, and associated increases in infectivity, are widespread among the ortho- and paramyxoviruses. Such processing can influence both virulence and host cell range (22). Bacterial plasminogen activators, in particular, influence the replicative cycle of influenza virus, with consequences for pathogenesis (41), and the virulence of *Yersinia pestis* (44).

It is particularly interesting that in previous experiments, the secretion of uPA by macrophages was found to be increased in the presence of HIV-1 virions that had been derived from prior culture in macrophages exactly as described here (15). In those experiments, uninfected macrophages exposed (under lipopolysaccharide-free conditions) to HIV-1 IIIB particles for just 1 h expressed uPA at levels that were severalfold higher than those in resting macrophages. Given that the association of gp120 with uPA or analogous proteinases may be critical to fusion of HIV-1 with these cells, our data suggest the existence of an infective cycle in which HIV-1, encountering its target cell, induces expression of uPA that is then used for fusion with the membrane. It follows that fusion is a target at which a proteinase inhibitor with appropriate specificity could be expected to act (31).

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REFERENCES

1. Andersen, K. B., and H. Skov. 1989. Retrovirus-induced cell fusion is enhanced by protease treatment. J. Gen. Virol. 70:1921–1927.
2. Anderson, E. D., L. Thomas, J. S. Hayflick, and G. Thomas. 1993. Inhibition of HIV-1 gp160-dependent membrane fusion by a furin-directed α_1-antitrypsin variant. J. Biol. Chem. 268:24887–24891.
3. Avril, L. E., M. Di Martino-Ferrer, G. Pignede, M. Séman, and F. Gauthier. 1994. Identification of the U-937 membrane-associated proteinase interacting with the V3 loop of HIV-1 gp120 as cathepsin G. FEBS Lett. 345:81–86.

4. **Berger, E. A., J. R. Sisler, and P. L. Earl.** 1992. Human immunodeficiency virus type 1 envelope protein glycoprotein molecules containing membrane fusion-impairing mutations in the V3 region efficiently undergo soluble CD4-stimulated gp120 release. J. Virol. 66:6208–6212.
5. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Lab. Clin. Invest. Suppl. 97:77–89.
6. **Cheng-Mayer, C., and J. A. Levy.** 1988. Distinct biological and serological properties of human immunodeficiency viruses from the brain. Ann. Neurol. 23:S58–S61.
7. **Clements, G. J., M. L. Price-Jones, P. E. Stephens, C. Sutton, T. F. Schulz, P. R. Clapham, J. A. McKeating, M. O. McClure, S. Thomson, M. Marsh, J. Kay, R. A. Weiss, and J. P. Moore.** 1991. The V3 loops of the HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: a possible function in viral fusion. AIDS Res. Hum. Retroviruses 7:3–16.
8. **Decroly, E., M. Vandenbranden, J. M. Ruysschaert, J. Cogniaux, G. S. Jacob, S. C. Howard, G. Marshall, A. Kompelli, A. Basak, F. Jean, C. Lazure, S. Benjannet, M. Chrétien, R. Day, and N. G. Seidah.** 1994. The convertases furin and PC1 can both cleave the human immunodeficiency virus (HIV)-1 envelope glycoprotein gp160 into gp120 (HIV-1 SU) and gp41 (HIV-1 TM). J. Biol. Chem. 269:12240–12247.
9. **Durda, P. J., L. Bacheler, P. Clapham, A. M. Jenoski, B. Leece, T. J. Matthews, A. McKnight, R. Pomerantz, M. Rayner, and K. J. Weinhold.** 1990. HIV-1 neutralizing monoclonal antibodies induced by a synthetic peptide. AIDS Res. Hum. Retroviruses 6:1115–1123.
10. **Ellis, V., N. Behrendt, and K. Danø.** 1991. Plasminogen activation by receptor-bound urokinase: a kinetic study with both cell-associated and isolated receptor. J. Biol. Chem. 266:12752–12758.
11. **Freed, E. O., E. L. Delwart, G. L. Buchschacher, and A. T. Panganiban.** 1992. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. Proc. Natl. Acad. Sci. USA 89:70–74.
12. **Freed, E. O., D. J. Myers, and R. Risser.** 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J. Virol. 65:190–194.
13. **Ghiara, J. B., E. A. Stura, R. L. Stanfield, A. T. Profy, and I. A. Wilson.** 1994. Crystal structure of the principal neutralization site of HIV-1. Science 264: 82–85.
14. **Hamilton, J. A., G. Vairo, K. R. Knight, and B. G. Cocks.** 1991. Activation and proliferation signals in murine macrophages. Biochemical signals controlling the regulation of macrophage urokinase-type plasminogen activator by colony-stimulating factors and other agents. Blood 77:616–627.
15. **Handley, M. A., S. A. Morrison, and R. T. Steigbigel.** 1992. Human immunodeficiency virus infection of macrophages induces secretion of protease. Clin. Res. 40:355A. (Abstract.)
16. **Handley, M. A., R. T. Steigbigel, and S. A. Morrison.** 1994. Urokinase (uPA) cleaves gp120 of HIV-1 and promotes viral infectivity. Clin. Res. 42:155A. (Abstract.)
17. **Harvima, I. T., R. J. Harvima, G. Nilsson, L. Ivanoff, and L. B. Schwartz.** 1993. Separation and partial characterization of proteinases with substrate specificity for basic amino acids from MOLT-4 T lymphocytes: identification of those inhibited by variable-loop-V3 peptides of HIV-1 envelope glycoprotein. Biochem. J. 292:711–718.
18. **Hattori, T., A. Koito, K. Takatsuki, H. Kido, and N. Katunuma.** 1989. Involvement of tryptase-related cellular protease(s) in human immunodeficiency virus type 1 infection. FEBS Lett. 248:48–52.
19. **Hoffman, A. D., B. Banapour, and J. A. Levy.** 1985. Characterization of the AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions. Virology 147:326–335.
20. **Hollinger, F. B. (ed.).** 1993. ACTG virology manual for HIV laboratories, p. Mac-1–Mac-4. Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, Md.
21. **Johnson, V. A., and R. E. Byington.** 1990. Quantitative assays for viral infectivity, p. 71–76. *In* A. Aldovini and B. D. Walker (ed.), Techniques in HIV research. Stockton Press, New York.
22. **Klenk, H.-D., and W. Garten.** 1994. Activation cleavage of viral spike proteins by host proteases, p. 241–280. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
23. **Kliks, S. C., T. Shioda, N. L. Haigwood, and J. A. Levy.** 1993. V3 variability can influence the ability of an antibody to neutralize or enhance infection by diverse strains of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 90:11518–11522.
24. **Koito, A., T. Hattori, T. Murakami, S. Matsushita, Y. Maeda, T. Yamamoto, and K. Takatsuki.** 1989. A neutralizing epitope of human immunodeficiency virus type 1 has homologous amino acid sequences with the active site of inter-α-trypsin inhibitor. Int. Immunol. 1:611–617.
25. **Koyanagi, Y., S. Miles, R. T. Mitsuyasu, J. E. Merrill, H. V. Vintners, and I. S. Y. Chen.** 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science 236:819–822.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
27. **Laman, J. D., M. M. Schellekens, G. K. Lewis, J. P. Moore, T. J. Matthews, J. P. Langedijk, R. H. Meloen, W. J. Boersma, and E. Claassen.** 1993. A hidden region in the third variable domain of HIV-1 IIIB gp120 identified by a monoclonal antibody. AIDS Res. Hum. Retroviruses 9:605–612.
28. **LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shadduck, L. H. Holley, M. Karpus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney.** 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science 249:932–935.
29. **Manchanda, N., and B. S. Schwartz.** 1990. Lipopolysaccharide-induced modulation of human monocyte urokinase production and activity. J. Immunol. 145:4174–4180.
30. **Manchanda, N., and B. S. Schwartz.** 1991. Single chain urokinase: augmentation of enzymatic activity upon binding to monocytes. J. Biol. Chem. 266:14580–14584.
31. **McNeely, T. B., M. Dealy, D. J. Dripps, J. M. Orenstein, S. P. Eisenberg, and S. M. Wahl.** 1995. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J. Clin. Invest. 96:456–464.
32. **Moore, J. P., B. A. Jameson, R. A. Weiss, and Q. J. Sattentau.** 1993. The HIV-cell fusion reaction, p. 234–289. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Inc., Boca Raton, Fla.
33. **Moore, J. P., Q. J. Sattentau, R. Wyatt, and J. Sodroski.** 1994. Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. J. Virol. 68:469–484.
34. **Murakami, T., T. Hattori, and K. Takatsuki.** 1991. A principal neutralizing domain of human immunodeficiency virus type 1 interacts with proteinase-like molecule(s) at the surface of Molt-4 clone 8 cells. Biochim. Biophys. Acta 1079:279–284.
35. **Myers, G., S. Korber, S. Wain-Hobson, K. T. Jeang, L. E. Henderson, and G. N. Pavlakis (ed.).** Human retroviruses and AIDS 1994. Los Alamos National Laboratory, Los Alamos, N.Mex.
36. **Nieva, J. L., S. Nir, A. Muga, F. M. Goñi, and J. Wilschut.** 1994. Interaction of the HIV-1 fusion peptide with phospholipid vesicles: different structural requirements for fusion and leakage. Biochemistry 33:3201–3209.
37. **Nuovo, G. J.** 1992. PCR *in situ* hybridization: protocols and applications. Raven Press, New York.
38. **Ou, C. Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. J. Sninsky, J. W. Krebs, P. Feorino, D. Warfield, and G. Schochetman.** 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 239:295–297.
39. **Page, K. A., S. M. Stearns, and D. R. Littman.** 1992. Analysis of mutations in the V3 domain of gp160 that affect fusion and infectivity. J. Virol. 66:524–533.
40. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224:497–500.
41. **Scheiblauer, H., M. Reinacher, M. Tashiro, and R. Rott.** 1992. Interactions between bacteria and influenza A virus in the development of influenza pneumonia. J. Infect. Dis. 166:783–791.
42. **Schulz T. F., J. D. Reeves, J. G. Hoad, C. Tailor, P. Stephens, G. Clements, S. Ortlepp, K. A. Page, J. P. Moore, and R. A. Weiss.** 1993. Effects of mutations in the V3 loop of HIV-1 gp120 on infectivity and susceptibility to proteolytic cleavage. AIDS Res. Hum. Retroviruses 9:159–166.
43. **Skinner, M. A., A. J. Langlois, C. B. McDanal, J. S. McDougal, D. P. Bolognesi, and T. J. Matthews.** 1988. Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. J. Virol. 62:4195–4200.
44. **Sodeinde, O. A., Y. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. D. Goguen.** 1992. A surface protease and the invasive character of plague. Science 258:1004–1007.
45. **Stephens, R. W., J. Pollanen, H. Tapiovaara, G. Woodrow, and A. Vaheri.** 1991. Stimulation of cell surface plasminogen activation by heparin and related polyionic substances. Semin. Thromb. Hemostasis 17:201–209.
46. **Suzuki, K., B. P. Craddock, N. Okamoto, T. Kano, and R. T. Steigbigel.** 1993. Poly A-linked colorimetric microtiter plate assay for HIV reverse transcriptase. J. Virol. Methods 44:189–198.
47. **Werner, A., and J. A. Levy.** 1993. Human immunodeficiency virus type 1 envelope gp120 is cleaved after incubation with recombinant soluble CD4. J. Virol. 67:2566–2574.

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(12) **United States Patent**
Rosenberg et al.

(10) **Patent No.: US 6,248,715 B1**
(45) **Date of Patent: Jun. 19, 2001**

(54) **METHOD OF TREATING A UROKINASE-TYPE PLASMINOGEN ACTIVATOR-MEDIATED DISORDER**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/438,745**

(22) Filed: **May 10, 1995**

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(60) Division of application No. 08/280,288, filed on Jul. 26, 1994, now abandoned, which is a continuation-in-part of application No. 08/070,153, filed on Jun. 1, 1993, now abandoned.

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(52) **U.S. Cl.** **514/12**; 514/2
(58) **Field of Search** 514/8, 12, 2, 825

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,751,180		6/1988	Cousens et al.	435/68
4,791,068		12/1988	Loskutoff et al.	436/518
4,835,255		5/1989	Weissman et al.	530/350
4,880,734		11/1989	Burke et al.	435/68
4,999,194	*	3/1991	Broeze et al.	435/215
5,112,755		5/1992	Heyneker et al.	435/215
5,219,569		6/1993	Blaber et al.	424/94.63
5,340,833	*	8/1994	Bridget et al.	514/443
5,376,547	*	12/1994	Kalyan et al.	435/215
5,389,538	*	2/1995	Tanabe et al.	435/215
5,416,006	*	5/1995	Blasi et al.	435/215

FOREIGN PATENT DOCUMENTS

64187/94	10/1994	(AU) .
69542/94	12/1994	(AU) .
WO 86/06100	10/1986	(WO) .
WO 92/07083	4/1992	(WO) .
WO 94/22464	10/1994	(WO) .

OTHER PUBLICATIONS

Bowie et al. (1990) Science 247: 1307–1310.*
Ballance et al., "A Hybrid Protein of Urokinase Growth–Factor Domain . . . " *Eur. J. Biochem 207(1)*: 177–183.*
Rabbani et al. "Structural Requirements for the Growth Factor Activity . . . " *J. Biol. Chem. 267(20)*: 14151–14156, (Jul. 1992).*
Corti et al. "Epitope Mapping of the Anti–Urokinase Monoclonal . . . " *Thromb. Haemostasis 62(3)*: 934–939, (Nov. 1989).*
Mazar et al, "Domain Analysis of Urokinase Plasminogen Activator . . . " *Fibrinolysis 6 (Suppl. 1)*: 49–55, (1992).*
Niedbala et al., "Regulation of Human Squamous Cell Carcimona . . . " *Cancer Comm. 2(9)*: 317–324, (Sep. 1990).*
Kobayashi et al., "Saturation of Tumour Cell Surface Receptors . . . " *Br. J. Cancer 67*: 537–544, (Mar., 1993).*
Appella et al., "The Receptor–Binding Sequence of Urokinase" *J. Biol. Chem. 262*(10): 4437–4440, Apr., 1987.
Brake, 1989, "Secretion of Heterologous Proteins Directed by the Yeast α–Factor Leader" *Yeast Genetic Engineering* pp. 269–280.
Schmitt et al., "Fluoescent Probes as Tools to Assess the Receptor for the Urokinase–Type . . . " *Seminars Thromb. Hemo. 17*(3):291–302, Jul., 1991.
Harlow et al., "Antibodies: A Laboratory Marvel" Cold Spring Harbor Laboratory, 1988, p. 513.
Ito et al., "Antibodies Against a Nonapeptide of Polyomarvirus Middle T Antigen: Cross–Reaction with a Cellular Protein(s)" *J. Virol. 48*(3):709–720, Dec., 1983.
Lowman et al., 1991, "Selecting High–Affinity Binding Proteins by Monovalent Phage Display" *Biochemistry 30*:10832–10838.
Matthews et al., 1993, "Substrate Phage: Selection of Protease Substrates by Monovalent Phage Display" *Science 260*:1113–1117.
Nagai et al., 1985, "Molecular Cloning of cDNA Coding for Human Preprourokinase" *Gene 36*:183–186.
Roldan et al., 1990, "Cloning and Expression of the Receptor for Human Urokinase Plasminogen Activator, a Central Molecule of Cell Surface, Plasmin Dependent Proteolysis" *EMBO J. 9*:467–474.
Barr et al., 1989, *Yeast Genetic Engineering* 269–280.
Frederick et al., Mar., 1990, "Glucose Oxidase from *Aspergillus niger*" *J. Biological Chemistry* 265(7):3793–3802.
Kingsman and Kingsman, 1990, Genetic Engineering, Blackwell Scientific Publications.

* cited by examiner

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(57) **ABSTRACT**

A method of treating an uPA-mediated disorder is disclosed, which comprises providing and administering an effective amount of polypeptide consisting essentially of the EGF-like domain of human uPA or active analog thereof.

13 Claims, No Drawings

PHARM COMP
NO ATF AS CLAIMED AA 1-48

METHOD OF TREATING A UROKINASE-TYPE PLASMINOGEN ACTIVATOR-MEDIATED DISORDER

RELATED APPLICATIONS

This application is a divisional of U.S. Ser. No. 08/280,288, filed Jul. 26, 1994, now abandoned, which is a continuation of U.S. Ser. No. 08/070,153, filed Jun. 1, 1993 now abandoned.

DESCRIPTION

1. Technical Field

This invention relates to the fields of cellular biology and protein expression. More particularly, the invention relates to peptide ligands of the urokinase plasminogen activator receptor, and methods for preparing the same.

2. Background of the Invention

Urokinase-type plasminogen activator (uPA) is a multi-domain serine protease, having a catalytic "B" chain (amino acids 144–411), and an amino-terminal fragment ("ATF", aa 1–143) consisting of a growth factor-like domain (4–43) and a kringle (aa 47–135). The uPA kringle appears to bind heparin, but not fibrin, lysine, or aminohex-anoic acid. The growth factor-like domain bears some similarity to the structure of epidermal growth factor (EGF), and is thus also referred to as an "EGF-like" domain. The single chain pro-uPA is activated by plasmin, cleaving the chain into the two chain active form, which is linked together by a disulfide bond.

uPA binds to its specific cell surface receptor (uPAR). The binding interaction is apparently mediated by the EGF-like domain (S. A. Rabbani et al., *J Biol Chem* (1992) 267:14151–56). Cleavage of pro-uPA into active uPA is accelerated when pro-uPA and plasminogen are receptor-bound. Thus, plasmin activates pro-uPA, which in turn activates more plasmin by cleaving plasminogen. This positive feedback cycle is apparently limited to the receptor-based proteolysis on the cell surface, since a large excess of protease inhibitors is found in plasma, including α_2 antiplasmin, PAI-1 and PAI-2.

Plasmin can activate or degrade extracellular proteins such as fibrinogen, fibronectin, and zymogens. Plasminogen activators thus can regulate extracellular proteolysis, fibrin clot lysis, tissue remodeling, developmental cell migration, inflammation, and metastasis. Accordingly, there is great interest in developing uPA inhibitors and uPA receptor antagonists. E. Appella et al., *J Biol Chem* (1987) 262:4437–40 determined that receptor binding activity is localized in the EGF-like domain, and that residues 12–32 appear to be critical for binding. The critical domain alone (uPA_{12-32}) bound uPAR with an affinity of 40 nM (about 100 fold less than intact ATF).

S. A. Rabbani et al., supra, disclosed that the EGF-like domain is fucosylated at Thr_{18}, and reported that fucosylated EGF-like domain (uPA_{4-43}, produced by cleavage from pro-uPA) was mitogenic for an osteosarcoma cell line, SaOS-2. In contrast, non-fucosylated EGF-like domain bound uPAR with an affinity equal to the fucosylated EGF-like domain, but exhibited no mitogenic activity. Non-fucosylated EGF-like domain competed for binding to uPAR with fucosylated EGF-like domain, and reduced the mitogenic activity observed. Neither EGF-like domain was mitogenic in U937 fibroblast cells.

Previously, it was suggested that an "epitope library" might be made by cloning synthetic DNA that encodes random peptides into filamentous phage vectors (Parmley and Smith, *Gene* (1988) 73:305). It was proposed that the synthetic DNA be cloned into the coat protein gene III because of the likelihood of the encoded peptide becoming part of pIII without significantly interfering with pIII's function. It is known that the amino terminal half of pIII binds to the F pilus during infection of the phage into *E. coli*. It was suggested that such phage that carry and express random peptides on their cell surface as part of pIII may provide a way of identifying the epitopes recognized by antibodies, particularly using antibody to affect the purification of phage from the library. Devlin, PCT WO91/18980 (incorporated herein by reference) described a method for producing a library consisting of random peptide sequences presented on filamentous phage. The library can be used for many purposes, including identifying and selecting peptides that have a particular bioactivity. An example of a ligand binding molecule would be a soluble or insoluble cellular receptor (i.e., a membrane bound receptor), but would extend to virtually any molecule, including enzymes, that have the sought after binding activity. Description of a similar library is found in Dower et al., WO91/19818. The present invention provides a method for screening such libraries (and other libraries of peptides) to determine bioactive peptides or compounds. Kang et al., WO92/18619 disclosed a phage library prepared by inserting into the pvIII gene.

However, both the pIII and pVIII proteins are expressed in multiple copies in filamentous bacteriophage. As a result, the phage are selected and amplified based on their avidity for the target, rather than their affinity. To overcome this problem, a method for monovalent (only one test peptide per phage) phage display has been developed (H. B. Lowman et al., *Biochem* (1991) 30:10832–38). To obtain monovalent display, the bacterial host is coinfected with the phage library and a large excess of "helper" phage, which express only wild-type pIII (and/or pVIII) and are inefficiently packaged. By adjusting the ratio of display phage to helper phage, one can adjust the ratio of modified to wild-type display proteins so that most phage have only one modified protein. However, this results in a large amount of phage having only wild-type pIII (or pVIII), which significantly raises the background noise of the screening.

DISCLOSURE OF THE INVENTION

One aspect of the invention is a method for producing non-fucosylated uPA EGF-like domain, particularly uPA_{1-48}.

Another aspect of the invention is non-fucosylated uPA_{1-48}, which is useful for inhibiting the mitogenic activity of uPA in cancer cells.

Another aspect of the invention is a method for treating cancer and metastasis by administering an effective amount of a non-fucosylated uPA EGF-like domain, particularly uPA_{1-48}.

Another aspect of the invention is a method treating a uPA-mediated disorder by administering a composition comprising an effective amount of a non-fucosylated polypeptide consisting of the EDF-like domain by instillation in the eye.

Another aspect of the invention is a method for pre-enriching a monovalent phage display mixture prior to screening for binding to a target, by providing a mixture of monovalent display phage and non-displaying phage, wherein the monovalent display phage display both a candidate peptide and a common peptide, the common peptide is identical for each monovalent display phage, and the candidate peptide is different for different monovalent display phage; and separating all phage displaying the common peptide from phage not displaying a common peptide.

MODES OF CARRYING OUT THE INVENTION

A. Definitions

The term "huPA" refers specifically to human urokinase-type plasminogen activator. The "EGF-like domain" is that portion of the huPA molecule responsible for mediating huPA binding to its receptor (uPAR). The EGF-like domain, sometimes called the growth factor-like domain ("GFD"), is located within the first 48 residues of huPA. The critical residues (essential for binding activity) have been localized to positions 12–32, although a peptide containing only those residues does not exhibit a binding affinity high enough to serve as a useful receptor antagonist.

The term "huPAR antagonist polypeptide" refers to a polypeptide having a sequence identical to the EGF-like domain of huPA (residues 1–48), or an active portion thereof. An "active portion" is one which lacks up to 10 amino acids, from the N-terminal or C-terminal ends, or a combination thereof, of the $huPA_{1-48}$ polypeptide, and exhibits a $K_d \leqq 5$ nM with huPAR. The term "active analog" refers to a polypeptide differing from the sequence of the EGF-like domain of $huPA_{1-48}$ or an active portion thereof by 1–7 amino acids, but which still exhibits a $K_d \leqq 5$ nM with huPAR. The differences are preferably conservative amino acid substitutions, in which an amino acid is replaced with another natually-occurring amino acid of similar character. For example, the following substitutions are considered "conservative": Gly↔Ala; Val↔Ile↔Leu; Asp↔Glu; Lys↔Arg; Asn↔Gln; and Phe↔Trp↔Tyr. Nonconservative changes are generally substitutions of one of the above amino acids with an amino acid from a different group (e.g., substituting Asn for Glu), or substituting Cys, Met, His, or Pro for any of the above amino acids. The huPAR antagonist polypeptides of the invention should be substantially free of peptides derived from other portions of the huPA protein. For example, a huPAR antagonist composition should contain less than 20 wt % uPA B domain (dry weight, absent excipients), preferably less than 10 wt % uPA-B, more preferably less than 5 wt % uPA-B, most preferably no detectable amount. The huPAR antagonist polypeptides also preferably exclude the kringle region of uPA.

The term "expression vector" refers to an oligonucleotide which encodes the huPAR antagonist polypeptide of the invention and provides the sequences necessary for its expression in the selected host cell. Expression vectors will generally include a transcriptional promoter and terminator, or will provide for incorporation adjacent to an endogenous promoter. Expression vectors will usually be plasmids, further comprising an origin of replication and one or more selectable markers. However, expression vectors may alternatively be viral recombinants designed to infect the host, or integrating vectors designed to integrate at a preferred site within the host's genome. Expression vectors may further comprise an oligonucleotide encoding a signal leader polypeptide. When "operatively connected", the huPAR antagonist is expressed downstream and in frame with the signal leader, which then provides for secretion of the huPAR antagonist polypeptide by the host to the extracellular medium. Presently preferred signal leaders are the Saccharomyces cerevisiae α-factor leader (particularly when modified to delete extraneous Glu-Ala sequences), and the ubiquitin leader (for intracellular expression).

The term "transcriptional promoter" refers to an oligonucleotide sequence which provides for regulation of the DNA→mRNA transcription process, typically based on temperature, or the presence or absence of metabolites, inhibitors, or inducers. Transcriptional promoters may be regulated (inducible/repressible) or constitutive. Yeast glycolytic enzyme promoters are capable of driving the transcription and expression of heterologous proteins to high levels, and are particularly preferred. The presently preferred promoter is the hybrid ADH2/GAP promoter described in Tekamp-Olson et al., U.S. Pat. No. 4,876,197 (incorporated herein by reference), comprising the *S. cerevisiae* glyceralde-hyde-3-phosphate dehydrogenase promoter in combination with the *S. cerevisiae* alcohol dehydrogenase II upstream activation site.

The term "host" refers to a yeast cell suitable for expressing heterologous polypeptides. There are a variety of suitable genera, such as Saccharomyces, Schizosaccharomyces, Kluveromyces, Pichia, Hansenula, and the like. Presently preferred are yeast of the Saccharomyces genus, particularly *Saccharomyces cerevisiae*.

The term "uPA-mediated disorder" refers to a disease state or malady which is caused or exacerbated by a biological activity of uPA. The primnary biological activity exhibited is plasminogen activation. Disorders mediated by plasminogen activation include, without limitation, inappropriate angiogenesis (e.g., diabetic retinopathy, corneal angiogenesis, Kaposi's sarcoma, and the like), metastasis and invasion by tumor cells, and chronic inflammation (e.g., rheumatoid arthritis, emphysema, and the like). Fucosylaied ATF is also mitogenic for some tumor cells (e.g., SaOS-2 osteosar-coma cells), which sometimes self-activate in an autocrine mechanism. Accordingly, the huPAR antagonist of the invention is effective in inhibiting the proliferation of uPA-activated tumor cells.

The term "effective amount" refers to an amount of huPAR antagonist polypeptide sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting inappropriate angiogenesis, limiting tissue damage caused by chronic inflammation, and the like. The precise effective amount for a subject will depend upon the subject's size and health, the nature and severity of the condition to be treated, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation based on the information provided herein.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The term "pre-enriching" refers to increasing the concentration of candidate phage in a monovalent phage display mixture by removing phage which do not have a candidate peptide. A "monovalent phage display mixture" is a mixture of phage containing recombinant phage and helper phage in a ratio such that most phage display at most one recombinant surface protein.

The term "common peptide" refers to a distinctive heterologous (not wild-type) peptide sequence which is displayed identically by all recombinant members of a phage (or other host) library. The common peptide is preferably an epitope recognized by a high-affinity antibody, which is not cross-reactive with any epitopes naturally occurring in the wild-type phage. The common peptide permits one to select all recombinant phage (having a common peptide and a random candidate peptide) as a set, and purify them away from non-recombinant (wild-type) phage. The presently preferred common peptide is Glu-Tyr-Met-Pro-Met-Glu.

B. General Method

The present invention relies on the fact that yeast do not fucosylate proteins upon expression, but are able to express properly folded, active uPA and fragments. One may employ other eukaryotic hosts in the practice of the invention as long as the host is incapable of fucosylating proteins, whether naturally or due to manipulation (e.g., genetic mutation or antibiotic treatment). Presently preferred hosts are yeasts,

particularly Saccharomyces, Schizosaccharomyces, Kluveromyces, Pichia, Hansenula, and the like, especially *S. cerevisiae*. Strains AB110 and MB2-1 are presently preferred.

The expression vector is constructed according to known methods, and typically comprises a plasmid functional in the selected host. The uPA sequence used may be cloned following the method described in Example 1 below. Variations thereof (i.e., active fragments and active analogs) may be generated by site-specific mutagenesis, imperfect PCR, and other methods known in the art. Stable plasmids generally require an origin of replication (such as the yeast 2μ ori), and one or more selectable markers (such as antibiotic resistance) which can be used to screen for transformants and force retention of the plasmid. The vector should provide a promoter which is functional in the selected host cell, preferably a promoter derived from yeast glycolytic enzyme promoters such as GAPDH, GAL, and ADH2. These promoters are highly efficient, and can be used to drive expression of heterologous proteins up to about 10% of the host cell weight. The presently preferred promoter is a hybrid ADH2/GAP promoter comprising the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter in combination with the *S. cerevisiae* alcohol dehydrogenase II upstream activation site.

The expression vector should ideally provide a signal leader sequence between the promoter and the huPAR antagonist polypeptide sequence. The signal leader sequence provides for translocation of the huPAR antagonist polypeptide through the endoplasmic reticulum and export from the cell into the extracellular medium, where it may be easily harvested. There are a number of signal leader sequences known that are functional in yeast. The yeast α-factor leader is presently preferred (see U.S. Pat. No. 4,751,180, incorporated herein by reference).

Alternatively, the vector may provide for integration into the host genome, as is described by Shuster, PCT WO92/01800, incorporated herein by reference.

Transformations into yeast can be carried out according to the method of A. Hinnen et al., *Proc Natl Acad Sci USA* (1978) 75:1929–33, or H. Ito et al., *J Bacteriol* (1983) 153:163–68. After DNA is taken up by the host cell, the vector integrates into the yeast genome at one or more sites homologous to its targeting sequence. It is presently preferred to linearize the vector by cleaving it within the targeting sequence using a restriction endonuclease, as this procedure increases the efficiency of integration.

Following successful transformations, the number of integrated sequences may be increased by classical genetic techniques. As the individual cell clones can carry integrated vectors at different locations, a genetic cross between two appropriate strains followed by sporulation and recovery of segregants can result in a new yeast strain having the integrated sequences of both original parent strains. Continued cycles of this method with other integratively transformed strains can be used to further increase the copies of integrated plasmids in a yeast host strain. One may also amplify the integrated sequences by standard techniques, for example by treating the cells with increasing concentrations of copper ions (where a gene for copper resistance has been included in the integrating vector).

Correct ligations for plasmid construction may be confirmed by first transforming *E. coli* strain MM294 obtained from *E. coli* Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of D. B. Clewell et al., *Proc Natl Acad Sci USA* (1969) 62:1159, optionally following chloramphenicol amplification (D. B. Clewell, *J Bacteriol* (1972) 110:667). Isolated DNA is analyzed by restriction mapping and/or sequenced by the dideoxy method of F. Sanger et al., *Proc Natl Acad Sci USA* (1977) 74:5463 as further described by Messing et al., *Nucl Acids Res* (1981) 9:309, or by the method of Maxam and Gilbert, *Meth Enzymol* (1980) 65:499.

huPAR antagonist polypeptides may be assayed for activity by methods known in the art. For example, one may assay competition of the antagonist against native uPA for cell surface receptor binding. Competition for the receptor correlates with inhibition of uPA biological activity. One may assay huPAR antagonist polypeptides for anti-mitogenic activity on appropriate tumor cell lines, such as the osteosarcoma cell line SaOS-2 described in the art. Inhibition of mitogenic activity may be determined by comparing the uptake of ^{3}H-T in osteosarcoma cells treated with the antagonist against controls. One may also assay huPAR antagonists for anti-invasive activity on appropriate tumor cell lines, such as HOC-1 and HCT116 (W. Schlechte et al., *Cancer Comm* (1990) 2:173–79; H. Kobayashi et al., *Brit J Cancer* (1993) 67:537–44).

huPAR antagonists are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. When used to treat tumors, it may be advantageous to apply the huPAR antagonist directly to the site, e.g., during surgery to remove the bulk of the tumor. Accordingly, huPAR antagonist may be administered as a pharmaceutical composition comprising huPAR antagonist in combination with a pharmaceutically acceptable excipient. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable excipients include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol®, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene® (Marion), Aquaphor® (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively, one may incorporate or encapsulate the huPAR antagonist in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet® minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care® (Allergan), Neodecadron® (Merck, Sharp & Dohme), Lacrilube®, and the like, or may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide a huPAR antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in *Remington's Pharmaceutical Sciences* (Mack Pub. Co.).

The amount of huPAR antagonist required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art. The appropriate dosage may be determined by one of ordinary skill by following the methods set forth below in the examples. As a general guide,

about 0.01 mg/Kg to about 50 mg/Kg huPAR antagonist administered i.v. or subcutaneously is effective for inhibiting tissue damage due to chronic inflammation. For treating corneal angiogenesis, huPAR antagonist may be administered locally in a gel or matrix at a concentration of about 0.001 mg/Kg to about 5 mg/Kg.

C. EXAMPLES

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(Cloning and Expression of $huPA_{1-48}$)

DNA encoding residues 1–48 of mature human uPA (huPA) was cloned into a yeast expression vector as a fusion with the yeast alpha-factor leader (αFl), under transcriptional control of a hybrid ADH2-GAP promoter. The αFl is described in Brake, U.S. Pat. No. 4,870,008, incorporated herein by reference. The hybrid ADH2-GAP promoter is described in Tekamp-Olson et al., U.S. Pat. No. 4,876,197, and Tekamp-Olson et al., U.S. Pat. No. 4,880,734, both incorporated herein by reference.

The gene encoding huPA was obtained by PCR using the following sense and nonsense primers:

5'-ATGCTAGATCTAATGAACTTCATCAGGTACCATCG-3' (SEQ ID NO:1), and

5'-CGATAGATCTTTATTTTGACTTATCTATTTCACAG-3' (SEQ ID NO:2).

Each of the above primers introduces a BglII site at the ends for cloning into the expression vector. Additionally, the sense strand primer introduces a KpnI site 14 nucleotides downstream from the signal peptide cleavage site, and the nonsense strand primer introduces a stop codon after Lys at position 48. The template DNA used was a clone of full length mature huPA in a yeast expression vector, as an alpha-factor fusion (pAB24UK300, consisting of the yeast shuttle vector pAB24 having a cassette inserted at the BamHI site, the cassette containing the ADH2-GAP hybrid promoter, the yeast ai-factor leader, the coding sequence for mature human uPA, and the GAP terminator, obtained from P. Valenzuela, Chiron Corporation) derived from a human kidney cDNA library (M. A. Truett et al., DNA (1985) 4:333–49). Polymerase chain reactions were carried out in 100 μL volumes with the following components: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1 μM each primer, 9 ng template plasmid, and 2.5 U Taq DNA polymerase. The reaction conditions were 94° C. for 1 min, followed by 37° C. for 2 min, then 72° C. for 3 min, for 30 cycles. Both the PCR fragment and a subcloning vector (pCBR, described by Frederik et al., *J Biol Chem* (1990) 265:3793) containing the yeast expression cassette were digested with BglII and then ligated together, after treatment of the pCBR vector with alkaline phosphatase. Once the subclone was obtained (pCBRuPAα13), the expression cassette was isolated via BamHI digestion and ligated into the yeast shuttle vector (pAB24) to yield pAB24α13uPA1–48.

The expression plasmid was transformed into *Saccharomyces cerevisiae* AB110 (MATα leu2-3-112 ura3-52 pep4-3 [cir]°) using the lithium acetate method (Ito et al., *J Bacteriol* (1983) 153:163), and selected for uracil prototrophy. The plasmid copy number was then amplified by growth on minimal media without leucine, containing 8% glucose to keep ADH2-GAP promoter-mediated expression repressed. High level expression of secreted $huPA_{1-48}$ was obtained with pAB24α13uPA1–48 transformants of AB110 grown in leu medium and inoculating at 1:10 into YEP 4% glucose medium. All yeast cultures were grown at 30° C., 275 rpm, for 96 hours.

Example 2

(Purification of $huPA_{1-48}$)

One liter of yeast supernatant was harvested by centrifuging the cells at 2600×g. Protein was concentrated from the supernatant by adding 70% ammonium sulfate, incubating for 1 hr at 4° C., and separating the protein precipitate by centrifuging at 11,000×g for 1 hr at 4° C. The protein pellets were resuspended in buffer containing 20 mM potassium phosphate, pH 7.0, 50 mM NaCl, and 1 mM EDTA. The suspension was dialyzed against the same buffer, with two changes of 4 L, overnight at 4° C. The entire dialysate was loaded onto a 1.8 L SEPHADEX® G-50 column at room temperature. Fractions were collected and monitored with UV at 254 nm, then pooled based on 16% Tris-Tricine SDS-PAGE (Novex) under non-reducing conditions. The peak fractions, containing monomeric $huPA_{1-48}$, were then loaded onto a 22 mm C18 reverse phase HPLC column (Vydac) and the protein eluted with a 0.6% gradient of acetonitrile containing 1% TFA. The major peak eluting at 33.5 minutes was collected and lyophilized. The purification yield is summarized in Table 1:

TABLE 1

Purification of $huPA_{1-48}$

Sample	Total Protein	Total Units[b]	Yield
Crude supernatant	~200 mg[a]	3.3×10^6	—
Ammonium sulfate	160 mg	2.0×10^6	60%
G50 Column	103 mg	1.3×10^6	42%
HPLC Purified	8.4 mg	7.4×10^5	22%

[a]Estimated protein concentration due to interference with BCA assay
[b]Unit = volume of crude sample required to inhibit binding of ^{125}I-ATF 50% in competition with biotinylated suPAR.

Example 3

(Characterization of $huPA_{1-48}$)

Purified $huPA_{1-48}$ was subjected to amino acid analysis and N-terminal sequencing, yielding the expected composition and sequence. The Edman degradation was performed through residue 20. A stoichiometric amount of threonine was observed at cycle 18, indicating that this residue was not modified by fucosylation, as is found for uPA purified from eukaryotic cells. The absence of post translational modification was later confirmed by electrospray mass spectrometry. The binding activity of the recombinant $huPA_{1-48}$ was determined using a radio-receptor binding assay.

Baculovirus-derived recombinant human urokinase receptor was expressed as a truncated, soluble molecule as described previously for mouse L-cells (Masucci et al., *J Biol Chem* (1991) 266:8655). The purified receptor was biotinylated with NHS-biotin, and immobilized at 1 μg/mL in PBS/0.1% BSA on streptavidin coated 96-well plates. Human uPA ATF (residues 1–135, obtained from M. Shuman, University of California, San Francisco) was iodinated using the Iodogen method (Pierce), and used as tracer. The ^{125}I-ATF was incubated at 100–500 pM with increasing amounts of $huPA_{1-48}$ in triplicate (100 pM-1 μM) for 2 hours at room temperature in 0.1% BSA/PBS in a total volume of 200 μL. The plates were then washed 3 times with PBS/BSA, and the remaining bound radioactivity determined. The apparent K_d observed for $huPA_{1-48}$ was 0.3 nM, comparable to that reported for ATF and intact uPA.

Example 4

(Construction of $huPA_{1-48}$ Muteins)

In order to efficiently analyze the features of $huPA_{1-48}$, we performed a series of mutagenesis experiments utilizing phage display. Attempts to employ the system described by Scott and Smith, *Science* (1990) 249:386–90, were not successful. However, the use of monovalent phage display, using a phagemid and helper phage as described by Lowman et al., *Biochem* (1991) 30:10832–38, did result in functional display of the protein domain. Finally, we employed an affinity epitope "tag" to reduce the fraction of phage bearing only wild-type pIII protein, reducing the background in panning experiments.

A.) Construction of Phagemids:

The starting materials were a phagemid construct (pGMEGF) comprising a human epidermal growth factor (hEGF) gene linked to the lac promoter, using pBLUESCRIPT (Stratagene) as the backbone. The polylinker region of the vector contained within a PvuII fragment was replaced by a cassette comprising a leader sequence from the photo-bacterial superoxide dismutase fused to a synthetic gene for hEGF, in turn fused to residues 198–406 of the M13 pIII gene. The sequence of the insert is shown in SEQ ID NO:3. A synthetic gene encoding human urokinase residues 1–48 was obtained from J. Stratton-Thomas, Chiron Corporation.

Fusion proteins were generated using PCR. A first set of primers EUKMPCR1 and EUKGPCR1 were used with primer EUKPCR2 to add epitope tags to $huPA_{1-48}$ at the N-terminus, and to add an amber codon (TAG) and a BamHI site within residues 249–254 of the pIII protein at the C-terminus.

EUKMPCR1: CTCATCAAGCTTTAGCGGACTACAAAGACGAT GACGATAAGAGC-AATGAACTTCATCAAG (SEQ ID NO:5);

EUKGPCR1: CTCATCAAGCTTTAGCCGAATACATGCCAATGG AAAGCAATGAAC-TTCATCAAG (SEQ ID NO:6);

EUKPCR2: CACCGGAACCGGATCCACCCTATTTTGACTTATC (SEQ ID NO:7).

The PCR reactions yielded primary products of the expected sizes, 204 and 197 bp.

A second set of primers, SRO1 and EUKCPCR1, were used with the EGF-containing phagemid construct as template. These primers added a BamI site at pIII residues 250–251 and amplified a fragment ending at the unique ClaI site at residues 297–299 of pIII.

SRO1: GAAATAGATAAGTCAAAATAGGGTGGATCCGGT TCCGGTGATTTTGATT-ATG (SEQ ID NO:8); and

EUKCPCR1: GAAACCATCGATAGCAGCACCG (SEQ ID NO:9).

This PCR reaction yielded a primary product of approximately 180 bp. The PCR reaction products were separated from unreacted primers by size exclusion chromatography (Chromaspin-100, Clontech), digested with restriction enzymes Hd3 and BamHI (set 1) or BamHI and ClaI (set 2), and isolated from a 2.5% agarose gel, using the MERMAID procedure (Bio-101). Each of the set 1 fragments were ligated with the C-terminal reaction 2 fragment, the ligations digested with Hd3 and ClaI, and the resulting fragments ligated into pGMEGF (digested with Hd3 and ClaI, dephosphorylated with alkaline phosphatase). The ligations were transformed into *E. coli* JS5 (Biorad) by electroporation. Strain JS5 overproduces lac repressor, and is sup0, preventing expression of the uPA_{1-48}-pIII fusion protein due to the amber stop codon between the uPA_{1-48} and pIII genes. Correct clones were identified by restriction analysis and confirmed by DNA sequencing. These steps yielded phagemids pHM1a (M1Flag-uPA_{1-48}) and pHM3a (Glutag-uPA_{1-48}). The DNA sequences of the fusion proteins in these phagemids are shown in SEQ ID NO: 10 and SEQ ID NO:12.

The phagemid containing a synthetic gene for uPA_{1-48} was constructed in the same vector by the following steps. The sequence of the synthetic gene is shown in SEQ ID NO: 14. Plasmid pCBRuPA (16 μg), a derivative of pCBR (Frederick et al., *J Biol Chem* (1990) 265:3793) containing this synthetic gene for uPA_{1-48}, inserted between the yeast α-factor leader and GAPDH terminator as a BglII fragment, was digested with SacI and ClaI, and adapted for phagemid expression using the following set of synthetic oligonucleotides:

SRO35: AGCTTTAGCGGAATACATGCCAATGGAAAGCAATGAGCT (SEQ ID NO: 16);

SRO36: CATTGCTTTCCATTGGCATGTATTCCGCTAA (SEQ ID NO: 17);

SRO37: CGATAAGTCAAAATAGGGTG (SEQ ID NO: 18); and

SRO38: GATCCACCCTATTTTGACTTAT (SEQ ID NO: 19).

Oligonucleotides SRO36 and SRO37 (250 pmol) were phosphorylated with polynucleotide kinase and annealed with equimolar amounts of oligos SRO35 and SRO38, respectively. The two annealed duplexes (125 pmol) were ligated overnight with the digested plasmid DNA, the ligase heat inactivated, and the ends phosphorylated with polynucleotide kinase. The DNA was run on a 6% polyacrylamide gel and the correct sized band (ca. 200 bp) was excised and isolated. The insert was ligated with plasmid pHM1a (digested with Hd3 and BamHI) and phosphatased, and the ligations transformed into *E. coli* JS5. The correct recombinants were identified by restriction analysis, and confirmed by DNA sequencing, yielding phagemid pHM3-3.

B.) Production and Panning of Phagemids:

To produce phagemid particles, DNAs were transformed into *E. coli* strain XL1-BLUE (Stratagene) by electroporation. This strain was used because it is supE44 (TAG codon encodes Gln), laciQ (overproduces lac repressor), and makes phage (F'+). Overnight cultures were grown in 2×YT broth containing 50 μg/mL amnpicillin and 10 μg/mL tetracycline (to maintain the F'). Cells were diluted 1:50 or 1:100 into the same media, grown for 20 minutes as 37° C. for 10 minutes at 225 rpm to enhance phage attachment, and then grown with normal agitation at 325 rpm overnight. Phage particles were then purified and concentrated by two successive precipitations with polyethylene glycol. The concentrations of phage present were determined by infection of *E. coli* XL1-blue and plating on L broth plates containing 50 μg/mL ampicillin.

To pan for binding phage particles, small tissue culture plates were coated either with anti-Glu antibody (R. Clark, Onyx Corporation) or streptavidin at 10 μg/mL in PBS overnight. Plates were then blocked with PBS containing 0.1% BSA. To the streptavidin plates was then added 1 μg/mL of biotinylated secreted human urokinase receptor obtained by recombinant baculovirus infection of *A. californica* Sf9 cells. After 2 hours at room temperature, the plates were again blocked with BSA, and phage (10^6–10^{10} cfu) were added in 1 mL of PBS/BSA. After incubation for 1 hour, non-specifically adhered phage were removed by washing (7×1 mL PBS/BSA), and the remaining phage eluted with 1 mL of 0.1 M glycine, pH 2.2, for 30 minutes. The eluted phage were immediately neutralized with 1 M Tris, pH 9.4, and stored at 4° C. overnight. The number of

phage eluted was determined by titering on *E. coli* XL1-blue on ampicillin plates. The procedure, where phage are first bound and eluted from the Glu-Ab plates and then panned against receptor plates, reduces the high background that would otherwise result from the large number of phage containing only wild type pIII: only phage containing an insert in pIII display an epitope tag and are selected on anti-Glu MAbs plates.

Table 2 shows that phagemids displaying uPA_{1-48} are specifically bound and eluted from immobilized urokinase receptor. Table 3 demonstrates that the phagemid which displays a Glu tag-uPA_{1-48} fusion is specifically retained by immobilized Glu Ab. Finally, Table 4 shows that a population of the Glu-uPA_{1-48} phagemid which has been specifically eluted from the Glu Ab plates, is retained with a much higher yield on urokinase receptor plates, than is the unenriched phagemid population.

TABLE 2

Panning on Immobilized Receptor

Sample	Phage/phagemid	Input[c]	% Yield -uPAR	% Yield +uPAR
1[a]	1a	9.4×10^9	0.0018	0.094
2[b]	3a	1.4×10^{10}	0.0014	0.08
3[c]	pGMEGF	1.3×10^{10}	0.0015	0.0012
4[d]	LP67 (control)	1.4×10^9	—	0.0099

[a]M1-FLAG-UPAELD-short pIII (pHM1a)
[b]Glu-tag-UPAELD-short pIII (pHM3a)
[c]M1-FLAG-EGF-medium long pIII (pGMEGF)
[d]LP67-control phage (Amp^r M13)
[e]ampicillin resistant colonies, in cfu

TABLE 3

Panning phage with Glu-Ab or suPAR

Sample	Phage/phagemid	Input[a]	% Yield suPAR[b]	% Yield GluAb
1	pHM1a	1.5×10^{10}	0.55%	0.003%
2	pHM3a	2.5×10^{10}	0.44%	0.048%
3	LP67 (control)	3.5×10^5	0.008%	—

[a]ampicillin resistant colonies, in cfu
[b]soluble uPA receptor

TABLE 4

Panning GluAb-unenriched and enriched phage on suPAR

Sample	Phage/phagemid	Input[a]	% Yield suPAR[b]	% Yield GluAb
1	pHM3a	2.7×10^7	0.85%	0.08%
2	pHM3a (enriched)	6×10^6	9.7%	3.3%
3	LP67 (control)	5.4×10^6	<0.04%	<0.02%

[a]ampicillin resistant colonies, in cfu
[b]soluble uPA receptor

These enriched phagemid pools are used for multiple mutagenesis strategies in order to identify improved uPA_{1-48} ligands with altered specificity or improved affinity. For example the region between residues 13 and 32 of human uPA has been implicated in receptor binding (E. Appella et al., *J Biol Chem* (1987) 262:4437–40). Key residues in the region from 19–30 can be easily mutated by replacing the region between the unique restriction sites KpnI and MunI.

In order to rapidly and quantitatively assess the binding affinities of the resulting uPA_{1-48} variants, relatively large quantities of properly folded proteins are required. Although this could be done by bacterial expression, using the phagemid constructs in a sup0 strain and inducing with IPTG, such a strategy yields relatively small amounts of protein in the periplasm. A second strategy is to express the variants in yeast, as described above for the wild type protein. To accomplish this we have constructed a yeast expression vector which enables us to move fragments encoding residues 4–48 of uPA_{1-48} in a single step from the phagemid vectors. This was accomplished as follows: Plasmid pAGαG, identical to pCBR except for a small deletion of an Xba fragment in the ADH2-GAPDH promoter, was digested with SacI, which cleaves once within the promoter, and then treated with Mung Bean nuclease which destroys the site. Subsequent religation yielded plasmid pAGαG-Sac. Digestion with BglII and treatment with alkaline phosphatase yielded a vector into which was ligated the BglII fragment corresponding to the synthetic gene for uPA_{1-48}. Transformation of *E. coli* strain HB101 to ampicillin resistance and restriction analysis yielded the correct clone. The 2.4 kB BamHI fragment from this plasmid (pAGαG-Sac1-48synth), containing the expression cassette, was isolated and ligated into pAB24, which had been treated with BamHI and alkaline phosphatase. The resulting plasmid has unique SacI and XhoI sites which can be used for transfer of the phagemid 1–48 genes. This is accomplished by digesting the phagemid with BamHI, treating with Mung Bean Nuclease, digesting with SacI and isolating the 145 bp fragment. The vector is digested with XhoI, treated with Mung Bean Nuclease, digested with SacI, and treated with alkaline phosphatase. Ligation then yields the correct recombinants in a single step in the yeast expression vector. Transformation of yeast strain AB110 then yields high levels of secreted 1–48 variants for analysis.

Using this construct, one can express a library of uPA variations for screening. Variations may be constructed by a variety of methods, including low-fidelity PCR (which introduces a large number of random point mutations), site-specific mutation, primer-based mutagenesis, and ligation of the uPA_{1-48} sequence (or portions thereof) to a random oligonucleotide sequence (e.g., by attaching $(NNS)_x$ to the uPA_{1-48} coding sequence, or substituting NNS for one or more uPA_{1-48} codons). Generation of random oligonucleotide sequences is detailed in Devlin, WO91/18980, incorporated herein by reference. Phage displaying uPA_{1-48} variants (having one or more amino acid substitutions) are screened according to the protocol described above (using, e.g., pHM3a as a positive control) and selected for improved binding.

Example 5

(Formulation of $huPA_{1-48}$)

$huPA_{1-48}$ formulations suitable for use in chemotherapy are prepared as follows:

A) Injectable Formulation:

$huPA_{1-48}$	7.0 mg
Na_2HPO_4 (0.5 M)	0.5 mL
mannitol (25%)	2.5 mL
sodium laureate (1%)	2.5 mL
pH	7.5
PBS qs	20 mL

This formulation is prepared following the procedure set forth in U.S. Pat. No. 4,816,440, incorporated herein by reference. The formulation is administered by parenteral injection at the site to be treated. The formulation is also

generally suitable for administration as eyedrops directly to the conjunctiva, or by intranasal administration as an aerosol. Alternatively, a concentrated formulation (e.g., reducing the phosphate buffered saline to 2 mL) may be used to fill an ALZET® minipump, and the minipump implanted at the site to be treated.

B) Ophthalmic Preparation:

$huPA_{1-48}$	1 mg
fibronectin	69 mg
albumin	37.5 mg
water qs	3.0 mL
HCl (0.01 M) qs	pH 4.0

This dosage form is prepared following the procedure set forth in U.S. Pat. No. 5,124,155, incorporated herein by reference. The fibronectin and albumin are dissolved in water to form a 3.0 mL solution, and HCl added to a pH of 4.0, causing the fibronectin to flocculate. The flocculent is filtered, and combined with the $huPA_{1-48}$. The mixture is then placed in a contact lens mold, and the mold closed for 30 min to form a corneal "shield" in the shape of a contact lens. The shield releases $huPA_{1-48}$ over a period of time, and is useful for preventing angiogenesis of corneal tissue following ophthalmic surgery.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 22

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCTAGATC TAATGAACTT CATCAGGTAC CATCG 35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGATAGATCT TTATTTTGAC TTATCTATTT CACAG 35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 953 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

-continued

(vii) IMMEDIATE SOURCE:
(B) CLONE: M1Flag-EGF-pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..903

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC        51
                           Met Asn Lys Ala Lys Thr Leu Leu Phe
                             1               5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCG GAC TAC AAA       99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Asp Tyr Lys
 10                  15                  20                  25

GAC GAT GAC GAT AAG AAT TCT GAC AGT GAA TGC CCG CTG AGC CAC GAC      147
Asp Asp Asp Asp Lys Asn Ser Asp Ser Glu Cys Pro Leu Ser His Asp
                 30                  35                  40

GGC TAC TGC CTG CAC GAC GGT GTT TGC ATG TAC ATC GAA GCT CTA GAC      195
Gly Tyr Cys Leu His Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp
             45                  50                  55

AAG TAC GCA TGC AAC TGC GTT GTT GGG TAC ATC GGT GAG CGC TGC CAG      243
Lys Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile Gly Glu Arg Cys Gln
         60                  65                  70

TAC CGA GAT CTT AAG TGG TGG GAA CTC CGT GGG CCC TTC GTT TGT GAA      291
Tyr Arg Asp Leu Lys Trp Trp Glu Leu Arg Gly Pro Phe Val Cys Glu
     75                  80                  85

TAT CAA GGC CAA TCG TCT GAC CTG CCT CAA CCT CCT GTC AAT GCT GGC      339
Tyr Gln Gly Gln Ser Ser Asp Leu Pro Gln Pro Pro Val Asn Ala Gly
 90                  95                  100                 105

GGC GGC TCT GGT GGT GGT TCT GGT GGC GGC TCT GAG GGT GGT GGC TCT      387
Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Glu Gly Gly Gly Ser
                110                 115                 120

GAG GGT GGC GGT TCT GAG GGT GGC GGC TCT GAG GGA GGC GGT TCC GGT      435
Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Gly
            125                 130                 135

GGT GGC TCT GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT      483
Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala
        140                 145                 150

AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC GCG CTA CAG TCT      531
Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser
    155                 160                 165

GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT      579
Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala
170                 175                 180                 185

ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT      627
Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly
                190                 195                 200

GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT      675
Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly
            205                 210                 215

GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT      723
Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro
        220                 225                 230

TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT AGC GCT GGT      771
Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly
    235                 240                 245

AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT      819
Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg
250                 255                 260                 265

GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT      867
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe
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                270                 275                 280

TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAATCATGCG          913
Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
            285                 290

CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA                         953
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 293 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
  1               5                  10                  15

Ser His Gln Ala Leu Ala Asp Tyr Lys Asp Asp Asp Asp Lys Asn Ser
             20                  25                  30

Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His Asp Gly
         35                  40                  45

Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys Val
     50                  55                  60

Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp Leu Lys Trp Trp
 65                  70                  75                  80

Glu Leu Arg Gly Pro Phe Val Cys Glu Tyr Gln Gly Gln Ser Ser Asp
                 85                  90                  95

Leu Pro Gln Pro Pro Val Asn Ala Gly Gly Gly Ser Gly Gly Gly Ser
            100                 105                 110

Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly
        115                 120                 125

Gly Gly Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp
    130                 135                 140

Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu
145                 150                 155                 160

Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp
                165                 170                 175

Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp
            180                 185                 190

Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly
        195                 200                 205

Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu
    210                 215                 220

Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu
225                 230                 235                 240

Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile
                245                 250                 255

Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu
            260                 265                 270

Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu
        275                 280                 285

Arg Asn Lys Glu Ser
    290
```

(2) INFORMATION FOR SEQ ID NO:5:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 953 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: M1Flag-EGF-pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..903

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC        51
                           Met Asn Lys Ala Lys Thr Leu Leu Phe
                             1               5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCG GAC TAC AAA       99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Asp Tyr Lys
 10                  15                  20                  25

GAC GAT GAC GAT AAG AAT TCT GAC AGT GAA TGC CCG CTG AGC CAC GAC      147
Asp Asp Asp Asp Lys Asn Ser Asp Ser Glu Cys Pro Leu Ser His Asp
                 30                  35                  40

GGC TAC TGC CTG CAC GAC GGT GTT TGC ATG TAC ATC GAA GCT CTA GAC      195
Gly Tyr Cys Leu His Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp
             45                  50                  55

AAG TAC GCA TGC AAC TGC GTT GTT GGG TAC ATC GGT GAG CGC TGC CAG      243
Lys Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile Gly Glu Arg Cys Gln
         60                  65                  70

TAC CGA GAT CTT AAG TGG TGG GAA CTC CGT GGG CCC TTC GTT TGT GAA      291
Tyr Arg Asp Leu Lys Trp Trp Glu Leu Arg Gly Pro Phe Val Cys Glu
     75                  80                  85

TAT CAA GGC CAA TCG TCT GAC CTG CCT CAA CCT CCT GTC AAT GCT GGC      339
Tyr Gln Gly Gln Ser Ser Asp Leu Pro Gln Pro Pro Val Asn Ala Gly
 90                  95                 100                 105

GGC GGC TCT GGT GGT GGT TCT GGT GGC GGC TCT GAG GGT GGT GGC TCT      387
Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Glu Gly Gly Gly Ser
                110                 115                 120

GAG GGT GGC GGT TCT GAG GGT GGC GGC TCT GAG GGA GGC GGT TCC GGT      435
Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Gly
            125                 130                 135

GGT GGC TCT GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT      483
Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala
        140                 145                 150

AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC GCG CTA CAG TCT      531
Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser
    155                 160                 165

GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT      579
Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala
170                 175                 180                 185

ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT      627
Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly
                190                 195                 200

GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT      675
Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly
            205                 210                 215

GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT      723
Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro
        220                 225                 230
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```
TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT AGC GCT GGT      771
Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly
    235                 240                 245

AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT      819
Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg
250                 255                 260                 265

GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT      867
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe
                270                 275                 280

TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAATCATGCG           913
Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
            285                 290

CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA                          953
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 293 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
  1               5                  10                  15

Ser His Gln Ala Leu Ala Asp Tyr Lys Asp Asp Asp Asp Lys Asn Ser
            20                  25                  30

Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His Asp Gly
        35                  40                  45

Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys Val
    50                  55                  60

Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp Leu Lys Trp Trp
65                  70                  75                  80

Glu Leu Arg Gly Pro Phe Val Cys Glu Tyr Gln Gly Gln Ser Ser Asp
                85                  90                  95

Leu Pro Gln Pro Pro Val Asn Ala Gly Gly Gly Ser Gly Gly Gly Ser
            100                 105                 110

Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly
        115                 120                 125

Gly Gly Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp
    130                 135                 140

Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu
145                 150                 155                 160

Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp
                165                 170                 175

Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp
            180                 185                 190

Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly
        195                 200                 205

Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu
    210                 215                 220

Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu
225                 230                 235                 240

Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile
                245                 250                 255
```

-continued

```
Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu
            260                 265                 270

Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu
        275                 280                 285

Arg Asn Lys Glu Ser
    290
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKMPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
CTCATCAAGC TTTAGCGGAC TACAAAGACG ATGACGATAA GAGCAATGAA CTTCATCAAG     60
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKGPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
CTCATCAAGC TTTAGCCGAA TACATGCCAA TGGAAAGCAA TGAACTTCAT CAAG          54
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKPCR2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
CACCGGAACC GGATCCACCC TATTTTGACT TATC                                34
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-continued

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAATAGATA AGTCAAAATA GGGTGGATCC GGTTCCGGTG ATTTTGATTA TG 52

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKCPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAAACCATCG ATAGCAGCAC CG 22

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 779 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: M1Flag uPA1-48 - pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..729

(ix) FEATURE:
(A) NAME/KEY: AA inserted by suppressor strain
(B) LOCATION: 79

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC        51
                           Met Asn Lys Ala Lys Thr Leu Leu Phe
                             1               5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCC GAC TAC AAA       99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Asp Tyr Lys
 10                  15                  20                  25

GAC GAT GAC GAT AAG AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT      147
Asp Asp Asp Asp Lys Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys
                 30                  35                  40

GAC TGT CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC      195
Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn
             45                  50                  55

ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA      243
Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu
         60                  65                  70

ATA GAT AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT      291
Ile Asp Lys Ser Lys Gln Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr
     75                  80                  85
```

-continued

```
GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT      339
Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp
 90                  95                 100                 105

GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT      387
Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala
            110                 115                 120

ACT GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC      435
Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly
        125                 130                 135

CTT GCT AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC      483
Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser
    140                 145                 150

CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT      531
Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn
155                 160                 165

TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT      579
Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro
170                 175                 180                 185

TTT GTC TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC      627
Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp
            190                 195                 200

AAA ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC      675
Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala
        205                 210                 215

ACC TTT ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG      723
Thr Phe Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys
    220                 225                 230

GAG TCT TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA      779
Glu Ser
235
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 235 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
  1               5                  10                  15

Ser His Gln Ala Leu Ala Asp Tyr Lys Asp Asp Asp Asp Lys Ser Asn
            20                  25                  30

Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr
        35                  40                  45

Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro
    50                  55                  60

Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys Gln Gly
65                  70                  75                  80

Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn
                85                  90                  95

Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp
            100                 105                 110

Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile
        115                 120                 125

Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala
    130                 135                 140
```

-continued

```
Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp
145                 150                 155                 160

Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser
                165                 170                 175

Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys
            180                 185                 190

Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly
        195                 200                 205

Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser
    210                 215                 220

Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 773 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Glu-tag uPA1-48 - pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..723

(ix) FEATURE:
(A) NAME/KEY: AA inserted by suppressor strain
(B) LOCATION: 77

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC        51
                           Met Asn Lys Ala Lys Thr Leu Leu Phe
                             1               5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCC GAA TAC ATG       99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Glu Tyr Met
 10                  15                  20                  25

CCA ATG GAA AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT      147
Pro Met Glu Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys
                30                  35                  40

CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC ATT CAC      195
Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His
            45                  50                  55

TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATA GAT      243
Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp
        60                  65                  70

AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT GAA AAG      291
Lys Ser Lys Gln Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys
    75                  80                  85

ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC      339
Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
 90                  95                  100                 105

GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT      387
Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp
                110                 115                 120

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT      435
Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala
```

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            125                 130                 135

AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG      483
Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met
        140                 145                 150

GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT      531
Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg
    155                 160                 165

CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC      579
Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val
170                 175                 180                 185

TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA      627
Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile
                190                 195                 200

AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT      675
Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe
            205                 210                 215

ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT      723
Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
        220                 225                 230

TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA               773
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
  1               5                  10                  15

Ser His Gln Ala Leu Ala Glu Tyr Met Pro Met Glu Ser Asn Glu Leu
             20                  25                  30

His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr Cys Val
         35                  40                  45

Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro Lys Lys
     50                  55                  60

Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys Gln Gly Gly Ser
 65                  70                  75                  80

Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly
                 85                  90                  95

Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys
            100                 105                 110

Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly
        115                 120                 125

Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly
    130                 135                 140

Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
145                 150                 155                 160

Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro
                165                 170                 175

Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr
            180                 185                 190

Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe
        195                 200                 205
```

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```
Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe
    210                 215                 220

Ala Asn Ile Leu Arg Asn Lys Glu Ser
225                 230
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 773 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Glu-tag uPA1-48 synth. - pIII map

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..723

(ix) FEATURE:
(A) NAME/KEY: AA inserted by suppressor strain
(B) LOCATION: 77

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC        51
                           Met Asn Lys Ala Lys Thr Leu Leu Phe
                             1               5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCG GAA TAC ATG       99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Glu Tyr Met
 10                  15                  20                  25

CCA ATG GAA AGC AAT GAG CTC CAT CAA GTA CCA TCG AAC TGT GAC TGT      147
Pro Met Glu Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys
                 30                  35                  40

CTA AAT GGA GGT ACC TGT GTG TCC AAC AAG TAC TTT TCG AAC ATT CAC      195
Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His
             45                  50                  55

TGG TGC AAT TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATC GAT      243
Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp
         60                  65                  70

AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT GAA AAG      291
Lys Ser Lys Gln Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys
     75                  80                  85

ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC      339
Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
 90                  95                 100                 105

GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT      387
Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp
                110                 115                 120

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT      435
Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala
            125                 130                 135

AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG      483
Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met
        140                 145                 150

GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT      531
Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg
    155                 160                 165

CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC      579
Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val
170                 175                 180                 185
```

-continued

```
TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA      627
Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile
                190                 195                 200

AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT      675
Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe
            205                 210                 215

ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT      723
Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
        220                 225                 230

TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA               773
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
  1               5                  10                  15

Ser His Gln Ala Leu Ala Glu Tyr Met Pro Met Glu Ser Asn Glu Leu
            20                  25                  30

His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr Cys Val
        35                  40                  45

Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro Lys Lys
    50                  55                  60

Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys Gln Gly Gly Ser
65                  70                  75                  80

Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly
                85                  90                  95

Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys
            100                 105                 110

Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly
        115                 120                 125

Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly
    130                 135                 140

Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
145                 150                 155                 160

Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro
                165                 170                 175

Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr
            180                 185                 190

Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe
        195                 200                 205

Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe
    210                 215                 220

Ala Asn Ile Leu Arg Asn Lys Glu Ser
225                 230
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTTTAGCG GAATACATGC CAATGGAAAG CAATGAGCT 39

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO36

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CATTGCTTTC CATTGGCATG TATTCCGCTA A 31

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO37

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGATAAGTCA AAATAGGGTG 20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO38

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GATCCACCCT ATTTTGACTT AT 22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 amino acids
(B) TYPE: amino acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly
1               5                  10                  15

Gly Thr Cys Val His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly
            20                  25                  30

Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn
        35                  40                  45
```

What is claimed is:

1. A method for treating a urokinase-type plasminogen activator (uPA)-mediated disorder, said method comprising:

(i) providing a composition comprising a non-fucosylated polypeptide consisting essentially of $huPA_{1-18}$ or an active peptide analog thereof, wherein said active peptide analog is a polypeptide differing from the sequence of $huPA_{4-18}$ by one to seven amino acids, which possesses substantially the same or greater binding affinity to huPAR as $huPA_{4-18}$, and

(ii) administering an effective amount of said composition to a patient having a uPA-mediated disorder.

2. The method of claim 1, wherein said uPA-mediated disorder is selected from the group consisting of metastasis, inappropriate angiogenesis, and chronic inflammation.

3. The method of claim 1, wherein said uPA-mediated disorder is selected from the group consisting of Kaposi's sarcoma, diabetic retinopathy, and rheumatoid arthritis.

4. The method of claim 1, wherein said composition is administered by instillation in the eye.

5. The method of claim 1, wherein said uPA-mediated disorder is metastasis.

6. The method of claim 1, wherein said uPA-mediated disorder is inappropriate angiogenesis.

7. The method of claim 6, wherein said inappropriate angiogenesis is corneal angiogenesis.

8. The method of claim 1, wherein said uPA-mediated disorder is chronic inflammation.

9. The method of claim 1, wherein said uPA-mediated disorder is Kaposi's sarcoma.

10. The method of claim 1, wherein said uPA-mediated disorder is diabetic retinopathy.

11. The method of claim 1, wherein said uPA-mediated disorder is rheumatoid arthritis.

12. The method of claim 1, wherein said polypeptine is $huPA_{1-48}$.

13. The method of claim 1, wherein said polypeptide possesses a K_d of about 0.3 nM.

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